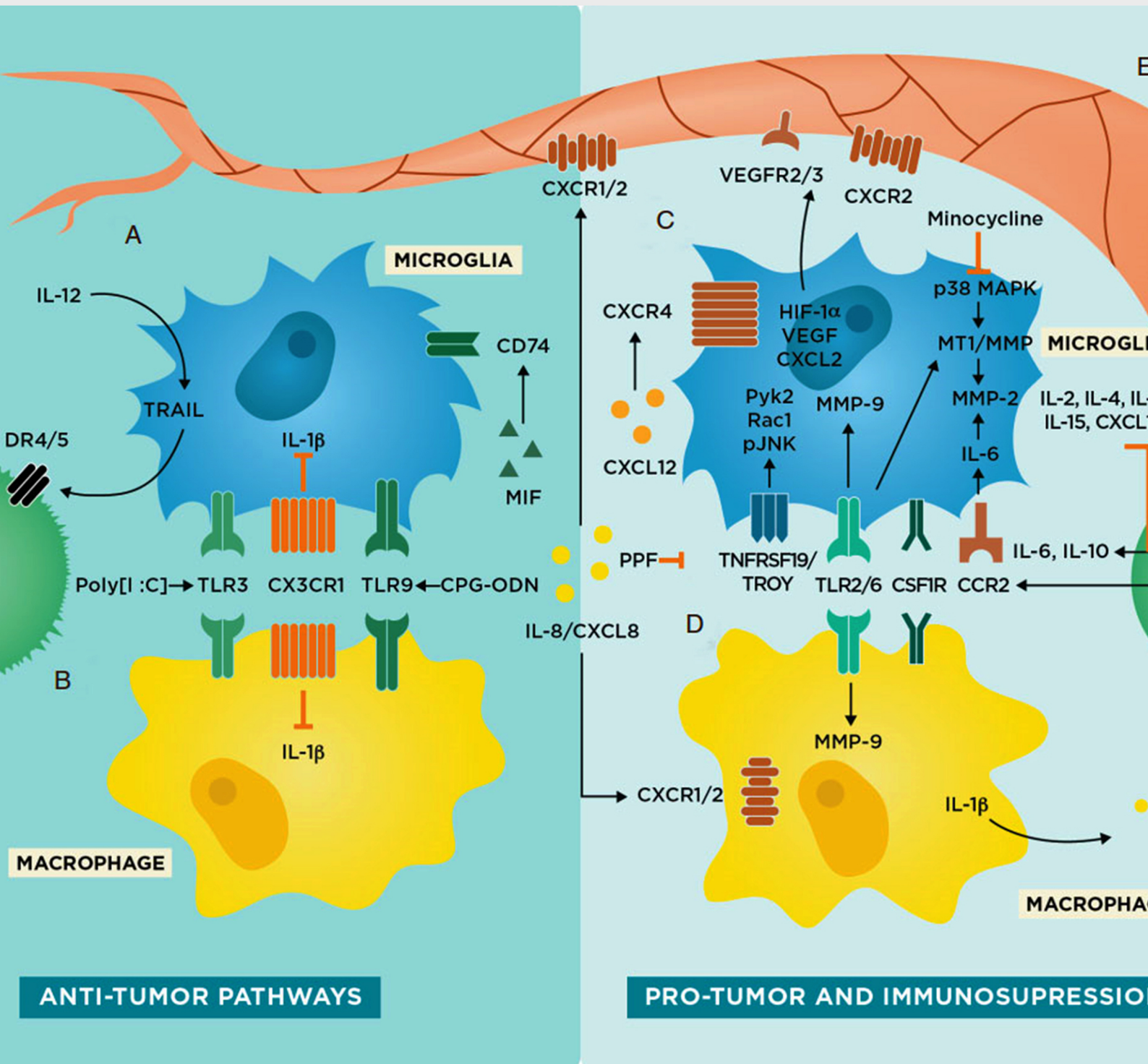


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Letter to Editor

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# Deep learning based computer-aided diagnosis for neuroimaging data: focused review and future potential

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Automatic image analysis techniques applied to neuroimaging data in general, and magnetic resonance imaging (MRI), and functional MRI (fMRI) in particular, have proven to be effective computer-aided diagnosis (CAD) tools in neuroscience<sup>[1-4]</sup>. Recently, the advancements in machine learning techniques combined with the wide availability of computational power have proven to be efficient in solving previously difficult problems in analyzing neuroimaging data. At the forefront of these advancements is the usage of deep (artificial) neural network architectures that led robust learning based techniques to attack challenging problems such as segmentation and classification in neuroimaging data<sup>[5-8]</sup>.

Many of the impressive results obtained in CAD using deep learning (DL) techniques utilize mainly image datasets. DL networks typically require annotations of several images for employing supervised learning techniques and are one of the roadblocks in employing these state of the art networks in various classification tasks in MRI/fMRI. However, unsupervised learning techniques within the DL paradigm are now being employed in natural image classification with a lot of success and we believe the adaptability of these to the neuroimaging data are required to attack challenging neuroimage analysis problems.

A stacked denoising auto encoders approach that is an unsupervised learning technique was used<sup>[9]</sup> for brain tumor segmentation in MRI imagery. The experimental results showed that using this particular approach is as good as using supervised learning based DL techniques that require accurate image-based annotations. This indicates that we can use different unsupervised learning in DL networks variants for



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various neuroimaging data problems. A Siamese DL networks approach<sup>[10]</sup> for detecting spinal metastasis with a multi-resolution technique correctly detected 100% of lesions on a dataset of 26 sagittal MR images from 14 males and 12 females ( $58 \pm 14$  years; mean  $\pm$  SD). The DL network considered produced only 0.40 false positives (FPs) per case. Further, at a true positive (TP) rate of 90%, with aggregation FPs were reduced from 0.375 FPs per case to 0.207 FPs per case obtaining 44.8% overall reduction. Although this work was for MR images of the spine, the usage of a Siamese neural network with the aggregation strategy promises to be an interesting approach that can also be adapted to brain MRI/fMRI imagery.

Utilizing domain-transfer convolutional neural networks, an end-to-end DL technique<sup>[11]</sup>, shows great promise since it overcomes the following problems of traditional classification and other DL based methods: (1) the need for manual design of feature space; (2) effective feature vector classifier or segment specific detection object and image patches; (3) large training datasets; (4) computing resources; and (5) long waiting times for training a perfect deep model. An example classification of the Open Access Series of Imaging Studies (OASIS)-MRI dataset showed good potential for such an approach's generalizability.

Extreme learning machines is a variant of DL networks, and an application in resting state fMRI data for schizophrenia was undertaken<sup>[12]</sup> and experimental results indicated that near 90% accuracy was obtained on a dataset of 72 patient images and 75 healthy controls (18 to 65 years) from the Center for Biomedical Research Excellence (COBRE)'s raw anatomical and fMRI data on this difficult classification problem. A DL pipeline<sup>[13]</sup> applied to recognize Alzheimer's disease using fMRI data obtained overall highest accuracy of 96.86% on 28 patient images and 15 healthy controls (24 female and 19 male,  $74.9 \pm 5.7$  years) from the Alzheimer's Disease Neuroimaging Initiative (ADNI) dataset.

Most of the CAD pipelines with DL techniques at their core utilize non-medical data to train due to the lack of availability of massive labeled data. Recent advancements in natural image analysis with DL methods are yet to be used for neuroimaging data and the challenges in obtaining the datasets/ annotations/labels, improvising/adapting DL networks, parameters setup, multi-modality generalization pose remain to be solved. However, the recent advancements in deep learning based image analysis shows great potential for analyzing MRI/fMRI imagery. Even with the limited results available so far in the literature, with deep learning based CAD for neuroimaging data we believe the future is bright for solving some of the hard neuroimage analysis problems.

## **DECLARATIONS**

### **Authors' contributions**

Prasath VBS contributed solely to this letter.

### **Financial support and sponsorship**

None.

### **Conflicts of interest**

There are no conflicts of interest.

### **Patient consent**

Not applicable.

### **Ethics approval**

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Review

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# Interactions between neurotropic pathogens, neuroinflammatory pathways, and autophagic neural cell death

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## Abstract

In recent times, there has been a significant increase in studies focusing on immunological functions of autophagy, however, knowledge of its roles and regulations in the central nervous system remains unclear. Present reviews highlight the molecular cross talk between host cell autophagy with inflammatory pathways in the context of neuro-infections. Intracellular pathogens might have an ability to manipulate the autophagy regulation process. An augmented autophagy and inflammation at the site of infection is traditionally considered host protective. Moreover, host cell autophagy might also facilitate pathogen survivability and multiplication in the brain environment. Consequently, an excessive autophagy and neuroinflammatory process do put surrounding healthy brain tissue at risk of pathogen invasion. The question arises, whether there are any known direct interactions of intracellular neurotropic pathogens with this degradative pathway that favour intracerebral pathogen survival and growth? It is worth exploring any such cooperation between pathogen factors and altered immune pathways that modulate autophagy regulatory genes causing massive neuronal damage. A detailed understanding of molecular mechanisms in microbial pathogenesis, neuroinflammatory and neuronal autophagy pathways might identify novel therapeutic targets and diagnostic biomarkers.

**Keywords:** Central nervous system infection, neurotropic pathogens, neuroinflammation, autophagy

## INTRODUCTION

Neurotropism of certain microbial pathogens could lead to neurological health problems in humans. It is suggested that chronic infections in the central nervous system (CNS) might be associated with progressive



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neurodegeneration and/or neurobehavioral abnormalities<sup>[1-4]</sup>. Prolonged CNS infections with neurotropic pathogens, along with other underlying conditions such as autoimmune responses, vascular diseases, head injury, cerebral edema, changes in neurotransmitter concentrations, nutritional deficiencies, heavy metal poisoning, and effects of environmental toxins, might play roles in the pathogenesis of neurodegenerative and/or neurobehavioral diseases<sup>[5-8]</sup>. However, the role of any CNS infections in specific pathogenesis of neuronal cell death has been not yet conclusively established.

Intracellular pathogens can invade host cells, protecting them from the host's systematic immune response<sup>[9]</sup>. However, they face a serious challenge within the infected cell, posed as competent hosts possess innate immune mechanisms specifically tailored to eradicate foreign invaders. To combat this, infectious agents follow one or more strategies in order to avoid host immune attack. These sophisticated strategies include: lysis and escape from the phagocytic compartment; modifying the phagosome microenvironment; surviving in acidic compartment. Some pathogenic agents might use more than one of the above strategies in order to successfully survive within the host cell<sup>[9]</sup>.

Presently, it is important to highlight interactions between the neurotropic pathogens and the host's immune system in the brain micro environment to understand the mechanism of pathogen survival mechanisms, and also the roles of host and pathogen factors in the pathogenesis of neurodegenerative and/or neurobehavioral diseases. Thus, it is interesting to revisit and update on innate immunity in the CNS, and also to further discuss the neuroinflammatory pathways which contribute to massive neurodegeneration in CNS infections by many different neuropathogens.

Recent studies have shown that pathogen induced autophagy negatively influences its replication, as shown in the case of Japanese encephalitis virus (JEV) infected mouse neuronal cells; autophagy has been found to delay virus-induced cell death<sup>[10]</sup>. On the other hand, for some neuropathogens (*Toxoplasma gondii* and other related parasites), an intrinsic role for autophagy has been identified in persistent infections<sup>[11]</sup>.

Besides autophagy being an intrinsic cellular defense mechanism against invading microorganisms, it can also appear to be linked to a number of other human disorders, such as autoimmune diseases, sterile inflammation, and even neoplasms<sup>[12]</sup>. Moreover, knowledge of its roles and regulations in the central nervous system remains unclear<sup>[13]</sup>.

The main purpose of the present review is to find out possible interactions of neurotropic intracellular pathogens with neuronal cell autophagic process favoring the pathogen(s) for their intracerebral survival, as well as multiplication in CNS infectious diseases.

## IMMUNE RESPONSES OF THE CENTRAL NERVOUS SYSTEM

The innate immunity of the CNS includes complex signaling pathways and a network of cells. Previously the brain was considered to be an immunologically privileged site in the human body. However, it is now known to have the ability to synthesize and release various active mediators, and a few pro-inflammatory molecules. The human brain also has the capacity to respond to any injury or insult, employing anti-inflammatory and/or pro-homeostatic mechanisms. Researchers at the University of Virginia, School of Medicine have discovered the presence of lymphatic vessels in the brain which connect the brain to body's immune system<sup>[14]</sup>.

A major part of CNS immunity revolves around the temporal relationship between cellular injury and the inflammatory response. Acute inflammation is the immediate response that occurs upon commencement of any injury or insult. The blood components, such as polymorphonuclear leukocytes from peripheral



circulation, have access to the CNS when the blood brain barrier (BBB) is compromised. In addition, with the latest discovery of lymphatic vessels between the meninges and skull bones, inflammatory mediators may have easier access to brain tissues. Even though these cells act as a defense system, they could contribute to cellular damage when excessively deployed in the interstitial space. Inflammation in the brain becomes chronic, and often pathological, when usually acute inflammation ceases in a short period and does not contribute to repair.

The microglia activation mechanism is an important determinant in the protection of the neural parenchyma in response to various infections, neuroinflammation, stroke, tumors, trauma, and neurodegenerative diseases<sup>[15]</sup>. Through a variety of mechanisms, activated microglia are the first cells in the CNS to respond to neuronal damage; they are usually able to exert two opposing functions both promoting neuronal regeneration, and killing neurons<sup>[15,16]</sup>. Exerting either function is largely determined by the particular conditions that evoke microglial activation<sup>[16]</sup>. However, the specific nature of any such constructive or destructive mechanisms remains nebulous.

Activated microglial cells release a number of cytotoxic molecules *in vitro*, (i.e. proteases, reactive oxygen intermediates, NO, cytokines, arachidonic-acid derivatives, excitatory amino acids, and quinolinic acid)<sup>[17-19]</sup>. HIV-infected mononuclear cells are known to produce low molecular weight neurotoxins, possibly causing neuronal damage via N-methyl-D-aspartate receptors<sup>[20]</sup>. The cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the CNS produced by microglial cells could cause bystander damage during the demyelination process. Moreover, the free oxygen radicals released by microglia have a direct toxic effect, as evident in co-cultures of neurons and microglia<sup>[21]</sup>. Mostly information on the cytotoxic properties of activated microglia are obtained from *in vitro* cultures, and has not yet been replicated *in vivo*. Furthermore, the cytotoxic properties of microglial cells are subject to considerable species variation; NO production is established for rat microglia, but in humans astrocytes might contribute to NO synthesis in addition to microglia<sup>[22]</sup>.

## AUTOPHAGY AND ITS REGULATING FACTORS UNDER PHYSIOLOGIC CONDITIONS

Autophagy is a cellular process that facilitates delivery of cytoplasmic constituents of eukaryotic cells for lysosomal degradation for nutrients recycling, and survival during starvation<sup>[23]</sup>. Physiologically, autophagy removes damaged or obsolete intracellular organelles. Meanwhile, it protects the body against microbial invasion by eliminating intracellular pathogens<sup>[24]</sup>.

Autophagy is induced by both metabolic and immune signals, comprised of pathogen recognition and proinflammatory cytokine mediated stimulation<sup>[25]</sup>.

There are three different types of autophagy known to occur in mammalian cells. (1) Macroautophagy-which relies on cytosolic double-membrane vesicle (autophagosomes) formation *de novo*, to sequester and transport cargo to the lysosome. (2) Chaperone-mediated autophagy-where individual unfolded proteins are transported directly across the lysosomal membrane. (3) Microautophagy- involving the direct uptake of cargo through invagination of the lysosomal membrane. All these types of autophagy ultimately lead to degradation of cargo and release of the breakdown products back into the cytosol for reuse by the cell<sup>[26]</sup>.

The degradative autophagy pathway is activated upon starvation, mediated through a protein kinase (Tor)<sup>[27]</sup>. This kinase might also inhibit the autophagy pathway, either by acting in a signal transduction cascade through various downstream effectors, or by causing the *ATG13* hyperphosphorylation<sup>[28]</sup>. Phosphorylated *ATG13* has a lower affinity for the *ATG1* kinase, and thereby a reduced interaction might inhibit the autophagy process<sup>[29]</sup>. The Gcn2, along with its targets, eIF2 $\alpha$ , and the Gcn4 transcriptional transactivator proteins in the downstream, might also play a role in induction of autophagy<sup>[30]</sup>.

Previously, studies on autophagy have been focused on its occurrences and association with nutrient deprivation, as well as age-associated issues. However, recently, there has been a significant increase in studies focusing on immunological functions of autophagy<sup>[12]</sup>. In the context of infection, immunity, and neurodegeneration, autophagy seems to have a pivotal role in neuronal homeostasis<sup>[31]</sup>. Its dysfunction has been linked to several neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's diseases. As the first line of defense in brain, the autophagic pathway is known to be involved in both physiological and pathological processes. However, its immunological role in the CNS environment is not yet clearly studied.

Interplay between inhibitory cytokines and the autophagy process has recently been reported, which reveals a novel mechanism by which autophagy could control the immune response. Interactions between autophagy and the regulatory cytokines IL-10, transforming growth factor- $\beta$ , and IL-27 are evident from earlier studies<sup>[12]</sup>. IL-37 and IL-35 are two newly discovered anti-inflammatory cytokines. IL-37 inhibits the production of pro-inflammatory cytokines in response to inflammation<sup>[32]</sup>. IL-35 suppresses T cell proliferation predominantly, and thereby inhibits its effector functions<sup>[33]</sup>. The possible interactions between above two anti-inflammatory cytokines, and autophagy have also been recently<sup>[12]</sup>. However, no such interaction in the context of the CNS environment is discussed in literature.

## **AUTOPHAGY INDUCTION AND ITS ALTERATION FOLLOWING INFECTION**

Accumulating evidence demonstrates that autophagy plays a protective role against infectious diseases by diminishing intracellular pathogens, including bacteria, viruses, and parasites. The following section will summarize the interplay between autophagy induction and infection due to various microbial agents.

Several intracellular bacterial agents, such as *Anaplasma phagocytophilum*, *Brucella abortus*, *Coxiella burnetii*, *Legionella pneumophila*, and *Staphylococcus aureus*, have the potential to alter the autophagic pathway to their own advantage<sup>[9]</sup>. These bacterial agents might stimulate their uptake and internalization into autophagosomes by secretion of special effector molecules<sup>[34]</sup>. Moreover, these pathogens seem to be efficiently grown within auto-phagosome compartments. Additionally, survival of some of these microorganisms is experimentally inhibited by using some autophagy inhibitors, and even their survival is compromised when grown within cells of deficient or defective ATG5 gene<sup>[35,36]</sup>.

Some studies have reported that some pathogenic bacteria also have ability to manipulate autophagy regulation processes at gene transcription level. For instance, *Francisella tularensis*, *Yersinia enterocolitica*, and *Burkholderia cenocepacia* can down-regulate the transcription of important autophagy-related (ATG) genes. Thus, they reduce the activity of autophagy at cellular level during infection. Conversely some pathogens up-regulate autophagy at gene level, which could augment inflammation at the site of infection. As previously discussed, prolonged inflammation could result in further injury to surrounding body tissues. In addition, the autophagic pathway is known to be exploited by RNA viruses (e.g. mouse hepatitis virus, rhinovirus and poliovirus), for promoting RNA replication with membrane scaffold<sup>[37]</sup>. In this review, the interactions between different CNS intracellular pathogens and autophagic genes, along with the resulting autophagic and inflammatory processes are being studied.

The presence of pathogenic antigens can induce autophagic genes through a stratified array of principal immunological processes, and therefore result in augmented autophagy and inflammation at the site of infection, which limits bacterial proliferation. However, as mentioned above, excessive autophagy and inflammatory process do put surrounding healthy brain tissue at risk.

A detailed understanding of molecular mechanisms in neuroinflammatory and neural cell death/autophagy dysfunction pathways might identify interesting targets for drug-discovery and biomarker identification in

various CNS disorders. Also, there is potential for precise anti-inflammatory drugs in future that may interfere with these genetic mechanisms, allowing more tailored therapeutics.

### Modulation of expression profiles of autophagic genes in response to infection

*Listeria Monocytogenes* is an intracellular Gram positive bacterium which can cause gastroenteritis. Upon its hematogenous dissemination it can breach BBB and thus cause meningitis, meningoencephalitis, and brain abscesses. An experiment using quantitative polymerase chain reaction (q-PCR) arrays containing 84 genes to analyse the expression profiles of autophagic genes in response to *L. monocytogenes* infection were carried out in rat brains. It was shown that 7 out of 84 genes were clearly modified with TNF, which functions to limit brain damage, being the most highly up-regulated gene after infection<sup>[38]</sup>. The expression of chemokine (C-X-C motif) receptor 4, and the insulin-like growth factor 1 that act as co-regulators of autophagy pathway, were down-regulated. Only the expression of core autophagy gene *ATG12* was modulated by infection.

During primary infection, intracellular *L. monocytogenes* along with infected host cells are usually targeted for degradation by autophagy. Once entering human body, *L. monocytogenes* are endocytosed into a vacuole, and listeriolysin O (LLO) is secreted to perforate the vacuole membrane, and subsequently gain access into the host cell cytosol. Subsequently, the product of the *actA* gene induces the polymerization of host actin, which eventually forms a tail, propelling bacterial movement within host cytosol for cell-to-cell spread. In this study of utilizing wild-type *L. monocytogenes* to infect mouse embryonic fibroblast cells, it is demonstrated that induction of protective autophagy pathway depends on expression of LLO, suggesting that vacuole permeabilization is a prerequisite for autophagy. On top of that, a mutant *L. monocytogenes* strain deficient of bacterial phospholipase production, has been degraded due to autophagy. It suggests that phospholipases plays a role in evasion of autophagy. Hence, it is hypothesized that the therapeutic target of *actA* as well as genes coding for phospholipases, might enhance autophagy, resulting in eradication of intracellular *L. monocytogenes*. Thus, identification of genes modulated upon infection in brain cells, as well as the mechanism of resulting autophagy, may confer a new strategy for therapeutic intervention in infectious disease<sup>[39-41]</sup>.

### Pathogen adaptations to host cell autophagy

Infectious agents which successfully parasitize their target cells have evolved to develop multiple strategies to dampen autophagy-dependent activation of host immune responses. So far, the types of identified microbial adaptation mechanisms can be broadly classified as strategies to: (1) prevent autophagy induction; (2) avoid pathogen recognition by the autophagic machinery; (3) prevent the autophagosome maturation into autolysosome; and (4) utilize components or functions of the autophagic mechanism to facilitate intracellular survival, multiplication, and release of intracellular microbes out of the infected cell<sup>[42]</sup>.

Herpes simplex viruses (HSVs) are able to inhibit autophagy in neurons, and subsequently confer neurovirulence by three main mechanisms, including the blockage of autophagy-stimulatory protein kinase resource (PKR) signaling, blockage of Beclin-1, or via the activation of mTOR signaling. In HSV-1, the utilization of a single viral virulence protein ICP34.5 to block the Beclin-1 dependent autophagy has been shown to be essential for HSV-1 encephalitis<sup>[43]</sup>. Of interest, it has been suggested that the inefficient fusion between lysosomes and autophagosomes in HSV-infected cells is caused by oxidative stress, which is reported to be associated with neurodegeneration<sup>[44]</sup>.

Coxsackievirus has been shown to utilize autophagosomes for replication, in which it limits autophagosome and lysosome fusion by increasing light chain 3 (LC3) cleavage. It has been hypothesized that this is achieved by increasing a protein-activated signaling cascade, known as the calpain-dependent pathway<sup>[45]</sup>.

A proposed mechanism of *L. monocytogenes* meningitis is that this intracellular pathogen camouflages and avoids recognition, subsequently rapidly replicating in the host cytosol by assembling host cell proteins to

the bacterial cell surface via ActA or InlK proteins. Also, *L. monocytogenes* phospholipase C is used to avoid autophagy by decreasing autophagic flux, diminishing host PI3P stores, and inhibiting the maturation of preautophagosomal structures<sup>[46]</sup>.

It is reported with evidence that autophagy plays a pivotal role as a major defense mechanism in host cells, not only in the brain, but in other cells as well. Recently, a number of reports have successfully elaborated the scope of the autophagic process in immunity, being evolved from an antimicrobial defense mechanism to a complex immunological process that plays a major role in adaptive immunity, innate immunity, and inflammation. In the support of the importance of autophagy as a key defense mechanism, it is now clear that highly evolved intracellular pathogens possess specialized anti-autophagic adaptations to block or hinder their elimination. Looking forward, it will be necessary to further understand how different microbes manipulate autophagy, and their interaction with host autophagy mechanisms, to provide a potential source for the development of antimicrobial treatment modalities that antagonize the pro-microbe responses, at the same time promoting the anti-microbe functions of autophagy.

### **Autophagy induction and intracellular replication in viral central nervous system infections**

There is a growing list of viruses which have been studied for relating their interactions with autophagy. For numerous viruses, there is a link between infection and autophagy. Certain viruses are responsible for the induction of an autophagic event, while others are involved in the intermediate processes of autophagy. Research has shown that autophagy not only plays a role in JEV and dengue infection, but also positively regulates the virus replication<sup>[47]</sup>. It is a well-known fact that energy is required for any cellular replication. In dengue viral infection, autophagy is shown to be connected to lipid metabolism in the virus, thus providing free fatty acids for synthesis of ATP<sup>[36]</sup>. This strongly suggests that the principle role of autophagy in dengue virus replication is for the regulating lipid metabolism. In contrast to dengue virus, the autophagic vacuole shows no evidence of being the site of replication for JEV. However, this finding is not consistent with the experiments conducted by Jin *et al.*<sup>[48]</sup> and Wang *et al.*<sup>[49]</sup>, who stated that the accumulation of the autophagosome and the autophagosome-lysosome fusion are essential to promote JEV replication<sup>[47-49]</sup>. In addition to initiating autophagy, viral intrusion also affects the intermediate step of the event. Evidence which supports this statement is the accumulation of p62 protein (which is degraded by autophagy process) in simian immunodeficiency virus (SIV)-infected brain tissues<sup>[50]</sup>.

It is also vital to find out if viral-induced autophagy will result in changes in the number of autophagosomes, which signifies the initiation of autophagy. Intriguingly, in the SIV-infected neurons, there is a significant reduction in the autophagosomes, in addition to changes in the distribution of the autophagosomes in the neurons<sup>[50]</sup>. The decrease in the number of autophagosomes may be due to the loss of neurons. This observation also implies the lack of initiation of autophagy in SIV infection. Further studies to test for the autophagic flux need to be carried out in order to determine how these two interact. Analysis showed that in SIV-infected microglia, there was loss of the neuronal processes and a decrease in the number of autophagosomes in the remaining processes; however the number of autophagosomes in the soma of the neurons remained constant. These findings demonstrate two reasonings: lack of initiation of the autophagy, and the increased clearance of the autophagosomes during the intermediate process. Further studies are required to analyze the importance of autophagosomal distribution in the pathogenesis of viral infection in microglia. It is interesting to note that rapamycin pre-treatment (an autophagy inducer) protected against the neurotoxic effects of the SIV, although the pre-treatment failed to recover the number of neurons to baseline level<sup>[50]</sup>. This indicates that autophagy might be one of the many pathways which serves to protect neurons in viral infections.

### **Increased hepatitis C virus replication and neurotoxicity with elevated autophagy**

Even though hepatitis C virus (HCV) is a hepatotropic virus, this infection is also considered a systemic disease with extra-hepatic manifestations. Up to 50% of patients with chronic HCV infection have

neuropsychiatric disorders. HCV RNA is detected in CD68+ cells of the CNS (macrophages/microglial) from 8 cases, suggesting direct HCV neuro invasion<sup>[51]</sup>. It is hypothesized that presence of lymphatic vessels in the brain might be the main pathway allowing infected peripheral blood monocytes to cross the BBB, and to serve as a precursors of the CNS microglia in addition to TNF- $\alpha$  and IL-8 secreted by microglial cells infected with HCV. These two proinflammatory cytokines are toxic to neurons. They mediate a local inflammatory response and are highly associated with neuropsychiatric disorders. Transcription factor NF- $\kappa$ B is reported to be involved in cytokine gene expression. Therefore, blocking NF- $\kappa$ B can be a therapeutic approach to controlling HCV mediated neuroinflammation<sup>[52]</sup>.

According to the above study, viral core proteins have been observed to activate microglial cells, astrocytes, and macrophages of patients infected with HCV. It has been shown that activation of microglial cells and subsequent diffusion of pro-inflammatory cytokines into the brain occurs as a result of changes in permeability of the BBB due to induced apoptosis of the brain microvascular endothelial cells in which the HCV virus replicates<sup>[53]</sup>.

Particularly, HCV proteins (core and non- structural) have been demonstrated to be involved in neurotoxicity in two ways.

(1) Direct exposure of primary human neurons to core proteins of HCV causes neurite retraction, leading to suppressed neuronal autophagy in the brain. It has been demonstrated that the level of LC3 is a marker of autophagosome formation. LC3 is highly expressed in human fetal neurons (HFN) exposed to Gal protein, while LC-II expression is significantly reduced in HCV core- exposed HFNs. It is suggested that HCV core proteins have the capability to inhibit LC3-I to LC3-II conversion, thereby reducing autophagosome formation in HCV core-exposed HFNs. Moreover, Gal-exposed neural cells demonstrated that HCV core proteins are active at directly at the neuronal membrane, contributing to the death of neurons by modulating the autophagy pathway<sup>[54]</sup>.

(2) HCV proteins activate by both toll-like receptor 2 (TLR2) signaling and extracellular signal-related kinase (ERK). Prolonged TLR2 mediated activation of ERK has been found to result in neuronal injury/ neurotoxicity.

In addition, a recent study reported that HCV triggers an unfolded protein response (UPR) and subsequently activates autophagy. Viral infection is often known to cause stress to endoplasmic reticulum (ER), and UPR (a signaling network) is specifically activated to restore ER homeostasis, refold misfolded proteins, and trigger the initiation of forming autophagosomes<sup>[55]</sup>. Consequently, it promotes the replication of HCV. However, under circumstances where accumulated unfolded and misfolded proteins in ER lumen are unresolvable, UPR can lead to apoptosis, resulting in chronic disorders such as neurodegeneration. In contrast, has also been reported that deficiency of *ATG7* can reduce the synthesis of infectious HCV virion particles without significant effects on the viral protein expressions and/or RNA biosynthesis<sup>[56]</sup>.

Moreover, according to the study, interference and subsequently loss of UPR-autophagy by gene silencing activates the innate immune response. It has been demonstrated that stable silencing of *ATG5*, or a UPR-activated transcriptional factor, CCAAT/enhancer binding protein homologous protein (CHOP) could further upregulate HCV pathogen-associated molecular pattern (PAMP)-triggered *interferon-beta* (*IFNB*) promoter activity and *IFNB* mRNA level. In addition, attenuated UPR-autophagy also remarkably elevated the downstream innate immunity pathways to inhibit replication of HCV in a paracrine fashion. Most importantly, it also demonstrated that a UPR-autophagy suppressing antiviral innate immune response can occur independently of this virus infection. Thus, interference in the UPR-autophagic pathway by gene modification exhibits therapeutic potential in suppressing viral replication<sup>[57]</sup>.



### Proviral role of autophagy in Japanese encephalitis virus and dengue virus infection

JEV is a mosquito-borne enveloped flavivirus with a positive-sense RNA genome, which causes acute encephalitis with high mortality in humans. Autophagy has been shown to be induced in human natural killer cells infected with an attenuated (RP-2ms) JEV strain, especially at the later stage, and to a lesser extent with a virulent (RP-9) strain<sup>[47]</sup>. In this study, the induction of autophagy by rapamycin was shown to enhance JEV replication, whereas 3-methyladenine mediated inhibition of autophagy reduced viral replication for both the strains of JEV. In addition, knockdown of *ATG5* or *Beclin 1* expression in cells also reduced JEV replication, suggesting a proviral role of autophagy in JEV multiplication. It has been shown that following endocytosis, the internalized JEV particles are targeted to preautolysosomal vacuoles (amphisomes) for viral uncoating. It is also hypothesized that an enhanced autophagy would increase the synthesis of viral RNA, leading to a raised viral protein expression level and yield of virus. In short, autophagy positively regulates JEV replication<sup>[47]</sup>.

Lately, the efficient dengue virus replication has been shown to be facilitated by an autophagy-dependent lipid droplets processing. Free fatty acids release, augmented cellular beta-oxidation, and ATP generation have provided energy and nutrient sources for viral production. It is noteworthy that dengue virus is also one of the members of Flaviviridae family<sup>[58]</sup>. In other words, therapeutic potential which inhibit autophagy upon infection has bacteriostatic effect too<sup>[59]</sup>.

### Contradictory findings of interaction between autophagy and HIV 1 virus in central nervous system infection

The autophagy in the brains of the HIV patients do not have any direct effect on neurons. However, neuronal dysfunction due to inflammation and the massive involvements of macrophages in HIV-induced encephalitis is apparent<sup>[60]</sup>. Wilcox *et al.*<sup>[61]</sup> reported a positive correlation of autophagy and apoptosis in the viral infected regions of neonatal brains, compared to adult brains which showed a negative relation in autophagy and apoptosis. This statement is supported by the observation made, that knock-out of *ATG7* in neonates resulted in decreased apoptosis<sup>[61]</sup>. Through this disconnection in the observations, it can be inferred that autophagic processes may be age dependent, particularly in the developing brains.

HIV destabilizes autophagy to facilitate self-replication, affecting genes essential for HIV replication (*ATG7*, *MAP1LC3B*, *ATG12* and *ATG16L2* involved in nucleation, and elongation of autophagosomes; *CLN3* and *LAPTM5* involved in lysosomal functioning) which are identified using small RNAi screening<sup>[62,63]</sup>. Studies elsewhere also demonstrated that replication of HIV is inhibited due to autophagy-associated silencing of genes: *BECN1* and *ATG5* in macrophages and *BECN1* and *ATG7* in monocytes. *BECN1* encodes for Beclin1 which is involved in regulation of autophagy in human body. HIV elicits autophagy activation but blocks the process of late proteolysis<sup>[62]</sup>.

Furthermore, in recent studies, HIV has been shown to inhibit autophagy in HIV-uninfected bystander cells. Also HIV *Tat* is known to inhibit interferon-gamma (IFN- $\gamma$ ) induced autophagy in macrophages that are uninfected by inhibiting *STAT1* phosphorylation. As a consequence, immune effector mechanism for targeted intracellular pathogens destruction is attenuated, and HIV patients are more susceptible to infections such as tuberculosis and toxoplasmosis<sup>[62]</sup>. In contrast to the effect of *Tat* on non-infected macrophages, HIV *Env* protein enhances apoptotic death of uninfected neurons and CD4+T cells, through a mechanism that involves accumulation of *BECN1* as well as autophagy induction<sup>[62]</sup>.

In the postmortem examination of the frontal cortices of both HIV-infected patients and non-infected individuals, autophagic markers were assessed by Western blotting and microscopy (confocal/electron). Autophagic proteins Beclin1, autophagy-related genes *ATG5*, *ATG7* and *LC-II* were observed to be significantly activated in brains from HIV-1 encephalitis cases. Autophagosome development needs Beclin1 for nucleation,

whereas covalent binding of *ATG5* to *ATG12* regulates vesicle elongation, following a pathway catalyzed by *ATG7* and *ATG10*<sup>[64]</sup>. In other words, the level of autophagy is likely increased in encephalitis caused by HIV. Additionally, autophagic protein levels and autophagosome formation are reported to be increased in the neurons of those treated with CXCR4- or CCR-tropic HIV-1 gp 120. In contrast, no increase in the extent of autophagic death is observed in the brains of non-encephalitic HIV patients compared to HIV-uninfected subjects. Therefore, the above study suggested that although autophagy could help neurons to sustain survival, the enhancement in autophagic death contributes to encephalitis and so also to cognitive impairment. Hence, the above study suggested that by combining drugs that reduce autophagic activity alongside anti-retroviral medications, the neurological deficits associated with HIV-1 infection could be prevented or reversed, since dysregulation of autophagy is highly correlated with pathogenesis of neurological HIV infection. Experiments in the future are needed to investigate further whether the process of autophagy leads to completion in the brains, or if there is any accumulation of autophagic proteins without autophagy-related degradation<sup>[62,65]</sup>.

### **Sindbis virus infection of central nervous system induces autophagy**

In this study, it has been demonstrated that Sindbis virus (SIN) infection induces autophagy in virally infected neurons *in vivo*, and viral antigen colocalizes with autophagosomes in neurons. It is important to note that *ATG* gene *ATG5* encodes an essential component of *ATG5-ATG12-ATG16* conjugation system, and is required for formation of autophagosomes<sup>[66]</sup>.

The role of the autophagy gene *ATG5* is very essential for providing protection against infection with lethal SIN in the mouse CNS. Inactivating *ATG5* in SIN-infected murine neurons results in a late viral protein clearance, an increased accumulation of adaptor protein p62, and enhanced neuronal death, however the viral replication levels remain unaffected. *In vitro*, the cellular protein p62 interacts with capsid protein of SIN and is required for capsid targeting to the autophagosome. Genetic deficiency of p62 blocks the targeting of viral capsids to autophagosomes, accelerates viral capsid aggregation, and thereby increases virus-induced cell death without hampering virus multiplication. In other words, increased SIN-induced animal mortality is not due to a direct role of neuronal *ATG5* in the control of viral replication or regulation of innate immune signaling. Rather, the disruption of neuronal *ATG5* function leading to neuronal death was actually associated with impaired SIN protein clearance. In this study, one plausible explanation is that in post mitotic cells such as neurons, the failure to properly clear viral proteins by autophagy results in cellular toxicity and increased animal lethality<sup>[67]</sup>.

### **Transmissible spongiform encephalopathies associated pathological implications of autophagy**

Though the autophagy process of cell death has been identified in scrapie (experimental infections) for quite some time this has only recently been reevaluated as a possible cell death process in prion diseases<sup>[68]</sup>. However, apoptosis is generally assumed to be the cause of neuronal death in transmissible spongiform encephalopathies (TSE)<sup>[69,70]</sup>. These pathologies of TSE, and many other neurodegenerative conditions, are characterized by the accumulation of aggregated and misfolded proteins in the brain; autophagy may be playing a protective role by removing such “toxic” protein aggregates<sup>[71,72]</sup>.

The scrapie responsive gene 1 (*Scrg1*) encodes a cysteine-rich protein that is highly conserved and expressed primarily in the CNS. This protein is targeted to the Golgi apparatus as well as large dense-core secretory granules in neuronal cells<sup>[68]</sup>. It has been recently demonstrated that the *Scrg1* protein is induced widely in scrapie-infected murine neurons, suggesting that *Scrg1* plays a role in the neuronal death and/or the host response to stress. The consistent association of autophagic structures (typical of scrapie) with *Scrg1* is in agreement with the recruitment of Golgi-specific proteins during this process of degradation, and therefore it is suggested that *Scrg1* might be used as a specific probe to identify the process of neuronal autophagy in TSE<sup>[68]</sup>.

## REGULATION OF AUTOPHAGY IN PERIPHERAL NEURONS

For decades, autophagy has been closely associated with both nutrient acquisition and pathogen destruction. In the apparent coevolution with pathogens, adaptive immune protection mechanisms of higher eukaryotic organisms such as human beings have also learned to exploit autophagy. Autophagy has evolved from a nutrient providing pathway to one which aids in higher functions in the innate, adaptive and cell-intrinsic immunological pathways.

Hence, it is not a surprise that autophagic pathways do indeed cross talk with innate immunity mechanisms, contributing to the combat of pathogenic infiltration. Though research into how pathogens obtain nutrients remains in its infancy, it has always been speculated that pathogens require autophagy as one of the many sources of intracellular nutrients, to survive and propagate in their host cells. All aspects considered, the critical questions that remain unanswered are, which nutrients do pathogens acquire from autophagy, and what is the degree to which they rely on these nutrients.

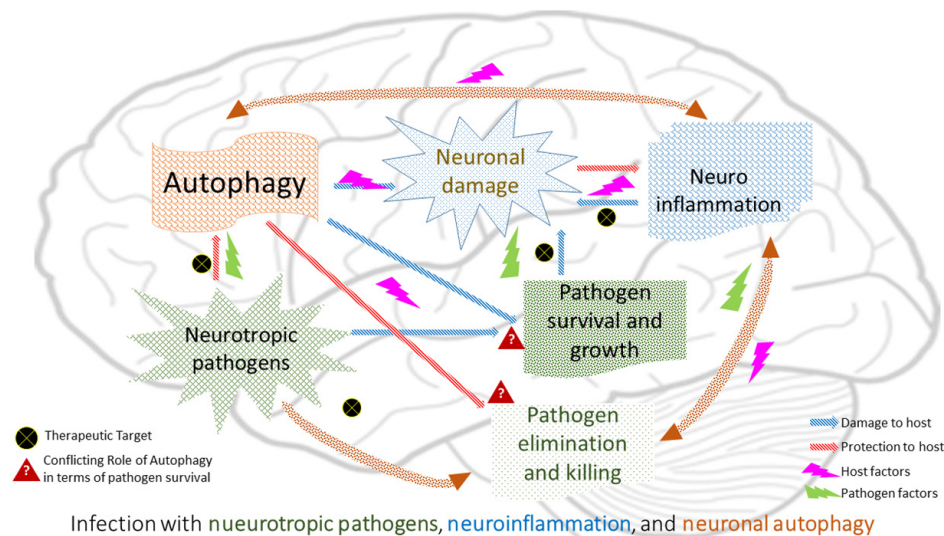
Neuronal autophagy is regulated uniquely, and is also adapted to a great extent along with local axonal physiology. Moreover, the detailed mechanism for neuronal autophagy might be significantly different to classically induced autophagy. In recent times, several studies have shown the importance of autophagy in various neurodegenerative conditions; studies have also identified autophagic process to be a potential target in drug discovery. Therefore, further understanding of the process of autophagic death of neuronal cells would eventually aid in novel drug target identification and rational designing of drug screening protocols in order to combat various neurodegenerative conditions<sup>[73]</sup>.

It is a good idea to identify some therapeutic agents that can control autophagy during infection, particularly when massive autophagic neural cell death causes the major pathology that might be induced possibly by some pathogen factors. Therapeutic manipulation of the interactions between autophagy and inhibitory cytokines might represent a novel method of regulating the immune response, and thereby a more facilitated clinical translation<sup>[12]</sup>.

Only very limited information is available on possible therapeutics to inhibit autophagy in terms of providing protection, particularly in the case of excessive autophagic death in neurodegenerative disorders. Inhibiting transglutaminase 2 (TG2), which is a multifunctional protein having implications in diverse pathophysiological processes, might offer a novel therapeutic approach for managing excessive autophagy<sup>[74]</sup>. Inhibition of the enhancer of zeste homolog 2 (EZH2) gene expression has also been shown to inhibit autophagy significantly, as reported in a study on human ovarian cancer where EZH2 expression could reverse the cisplatin resistance by inhibiting autophagy<sup>[75]</sup>. Downregulation of the methionine synthase reductase (MTRR) gene might also be an approach to inhibit the PI3K/Akt autophagy pathway; in a recent study, MTRR silencing could significantly increase cisplatin-induced apoptosis and reduce the autophagy induced by cisplatin in SKOV3/DDP cells<sup>[76]</sup>. The glucose regulated protein 78 (GRP78) is also known to affect autophagy and apoptosis; particularly in ovarian cancer cells. GRP78 is reported to have a regulatory role in expressions of *Beclin1*, *Bcl-2* and *CHOP*, thereby affecting the sensitivity to cisplatin in ovarian carcinoma, which may be a new method for ovarian carcinoma treatment through improvement of the sensitivity to cisplatin. However, there is no study done to validate such hypotheses regarding the possible role of inhibiting excessive neurodegeneration due to autophagy in minimizing the bulk self-digestion of the neuronal cells.

## CONCLUSION AND RECOMMENDATION

The presence of antigenic stimuli of pathogens can induce autophagic genes through a stratified array of principal immunologic processes, and therefore result in augmented autophagy and inflammation at the



**Figure 1.** Schematic presentation depicting possible cooperation between pathogen factors altering immune inflammatory pathways in general influencing host cell autophagy regulatory genes (hypothetical) that cause a massive neuronal damage in certain intracranial infections

site of infection, which is considered to be protective to the host. However, an excessive auto-degeneration of the neuronal cells can be harmful. The question arises, whether there are any known direct interactions of intracellular pathogens (having neurotropism) with this degradative pathway that favor the pathogens for intracerebral survival and growth? It is worth exploring if there is any cooperation between pathogen factors altering immune inflammatory pathways, thereby influencing host cell autophagy regulatory genes that cause massive neuronal damage in intracranial infections as hypothesized presently [Figure 1]. Targeting some key pathways with respect to infectious causes of neurodegeneration will be the need of tomorrow's new drug discovery that may or may not include the targeting of autophagy for minimizing brain matter degeneration.

## DECLARATIONS

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### Authors' contributions

Conceptualized the theme, designed the literature review process: Sahu PS

Contributed in the acquisition, analysis, and interpretation of information; drafted the manuscript; and approved the final version to be published: Sahu PS, Ter E

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### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

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Not applicable.

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Case Report

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# Progressive multifocal leukoencephalitis in a patient with sarcoidosis on hydroxychloroquine with negative cerebrospinal fluid testing for the John Cunningham virus

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## Abstract

Progressive multifocal leukoencephalopathy (PML) is a severe demyelinating disease of the central nervous system caused by the John Cunningham (JC) virus typically seen in immuno-compromised patients. Several drugs that suppress that immune system have already been known to cause PML such as natalizumab and rituximab. We present a patient with sarcoidosis who develops PML in the rare setting of minimal immunosuppression with only hydroxychloroquine. There was significant delay in the diagnosis due to negative cerebrospinal fluid testing for JC virus and concern for neuro-sarcoidosis, but eventually a diagnosis of PML was made via brain biopsy.

**Keywords:** Progressive multifocal leukoencephalopathy, sarcoidosis, hydroxychloroquine, neuroimmunology

## INTRODUCTION

Progressive multifocal leukoencephalopathy (PML) is a severe demyelinating disease of the central nervous system caused by the John Cunningham (JC) virus. Approximately 86% of adults have antibodies to the JC virus, believed to be secondary to an asymptomatic childhood infection<sup>[1]</sup>. When a patient becomes



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immunosuppressed, the JC virus can spread to the central nervous system, and cause a life-threatening severe neurological illness.

Patients with PML usually have gradually worsening neurological deficits including encephalopathy, motor deficits, and visual symptoms. PML lesions are generally in the white matter, and magnetic resonance imaging (MRI) typically shows that these lesions have no mass effect and no contrast enhancement. Diagnosis can be established by confirming JC virus DNA in the cerebrospinal fluid (CSF) along with typical imaging, but definitive diagnosis requires brain biopsy with the histopathologic triad of demyelination, bizarre astrocytes, and enlarged oligodendroglial nuclei coupled with techniques to show the presence of JC virus<sup>[2]</sup>. PML is an incredibly rare condition. It occurs almost always in patients with a suppressed immune system, either from medications or a preexisting illness such as human immunodeficiency virus (HIV), chronic lymphocytic leukemia, or lymphoma. Several immune suppressing medications have been known to cause PML, most notably natalizumab and rituximab. Hydroxychloroquine (also known as plaquenil) is a medication frequently used to suppress the immune system that has not been previously associated with PML.

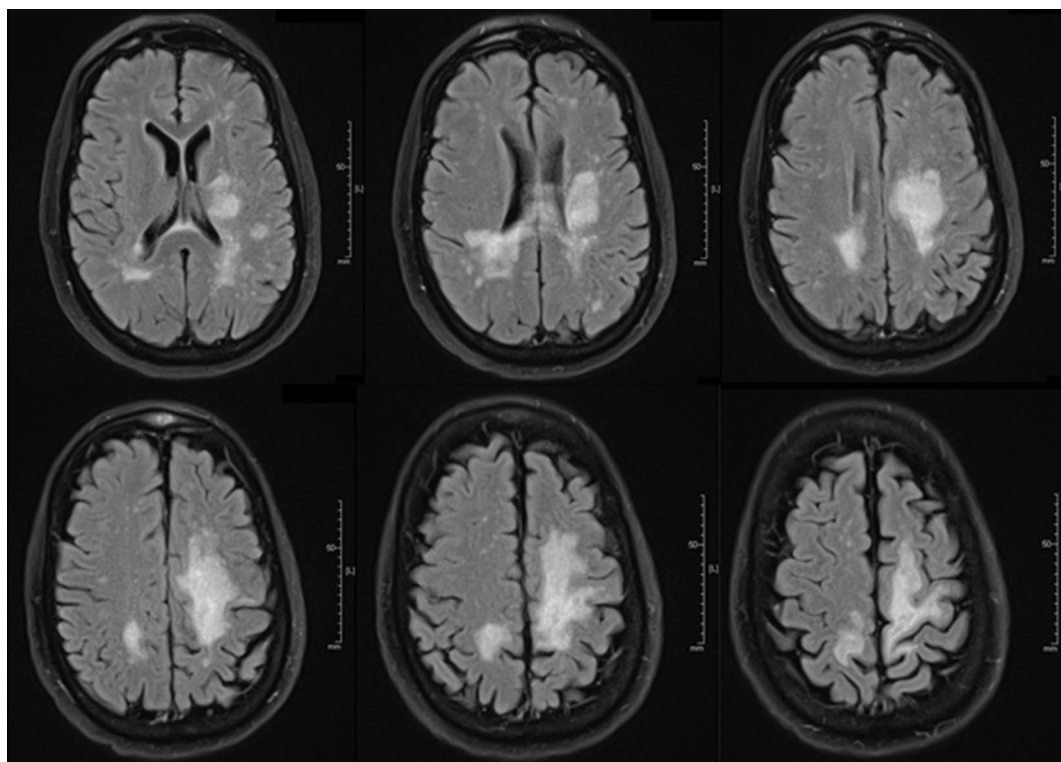
## CASE REPORT

Patient D is a 65-year-old man with a history of asthma, benign prostatic hyperplasia, pre-diabetes, and dermatologic sarcoidosis who presented to the Duke General Neurology service in July of 2017 with about 6 months of rapidly worsening right-sided weakness, facial weakness, and cognitive decline. He had been taking hydroxychloroquine 200 mg daily to treat his dermatologic sarcoidosis since September of 2014 (about 3 years prior to presentation). No other immunosuppressive drugs have been shown in medical records. His other home medications were paxil, aspirin, vitamin D, montelukast, and flomax. His rheumatologist also gave him a prolonged steroid taper after his symptoms first began.

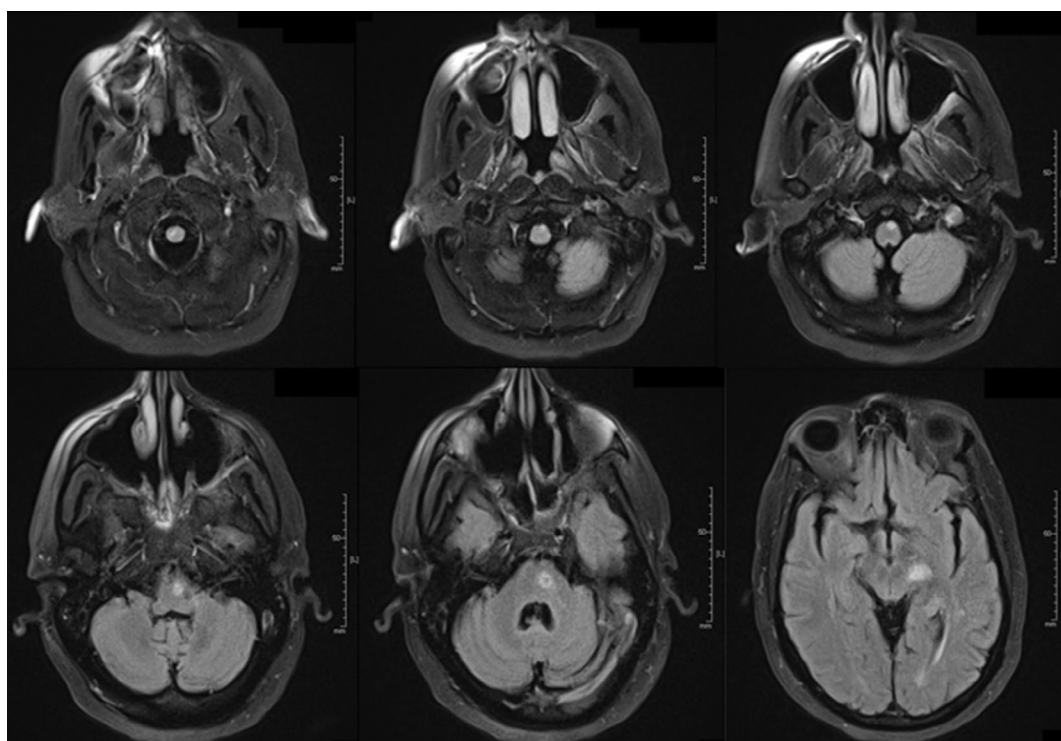
In mid-March, he initially noticed right foot numbness and weakness and went to his podiatrist and primary care doctor, who both documented normal neurologic examinations. In early April, the patient was seen by his rheumatologist, who reported right foot drop, prescribed an oral steroid course, and referred him to a neurologist, who documented weakness and decreased sensation to pinprick in the distal right lower extremity. In early May, he was noted to have involvement of his proximal right lower extremity, with mild weakness of hip flexion and extension, moderate weakness of knee flexion and extension, and almost complete inability to dorsiflex or plantarflex his right foot. In early July, he followed up with his neurologist and reported continued progression of weakness, including his right arm. On examination, he had a right pronator drift with mild weakness of his right hand intrinsic muscles and difficulty with fine motor skills, as well as mild weakness of hip flexion, moderate weakness of knee flexion and extension, and severe weakness of dorsiflexion and plantarflexion on his right side. He was walking with a walker and dragging his right foot, and he had fallen several times. He was also noted to have flattening of his right nasolabial fold.

Due to his multifocal and rapidly progressive motor symptoms, his preliminary diagnosis was a variant of motor neuron disease. He initially received a broad laboratory work-up, which was unimpressive including negative testing for HIV. Next, due to concern for motor neuron disease, electromyography was performed and showed increased insertional activity in the majority of muscle groups tested with generalized reduction in activation, concerning for a central nervous system etiology. Lumbar puncture was performed and was unremarkable, including negative JC virus polymerase chain reaction (PCR). MRI's of the cervical, thoracic, and lumbar spines were normal. Brain MRI performed revealed enlargement of non-enhancing subcortical and periventricular T2 hyperintensities [Figure 1] with Wallerian degeneration [Figure 2].

There was concern for neuro-sarcoidosis due to his history of dermatologic sarcoidosis despite the lack of contrast enhancement on the MRI lesions. Computed tomography scan of the chest revealed supraclavicular,



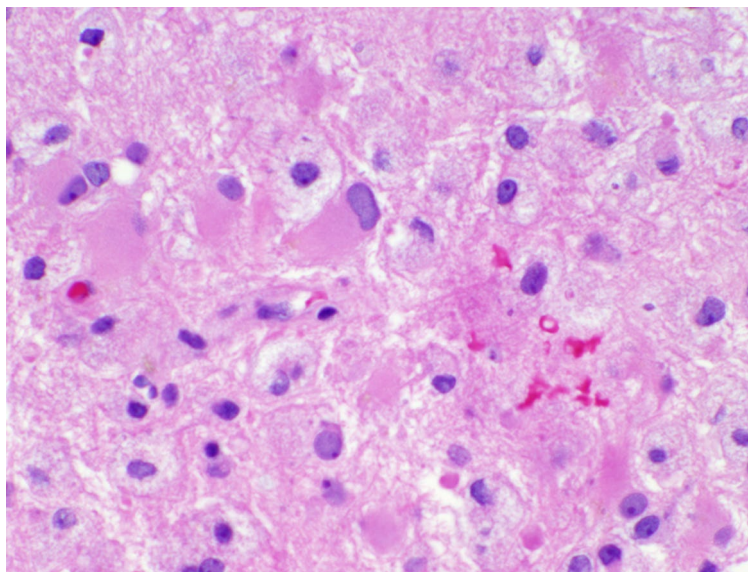
**Figure 1.** Fluid-attenuated inversion recovery brain magnetic resonance imaging (8 Sep 2017). Confluent periventricular and subcortical white matter lesions seen bilaterally



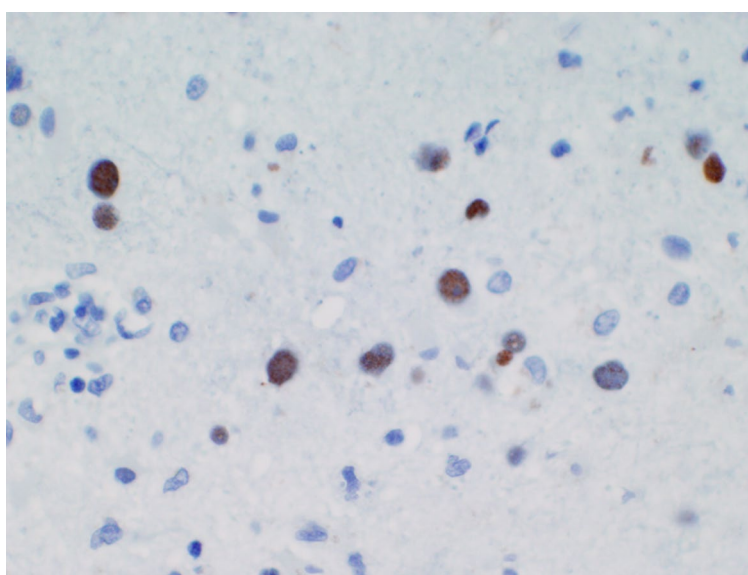
**Figure 2.** Fluid-attenuated inversion recovery brain magnetic resonance imaging (8 Sep 2017). Prominent Wallerian degeneration noted with lesion located in motor tract starting in the posterior limb of the internal capsule and extending inferiorly, decussating in the inferior medulla

mediastinal, and hilar lymphadenopathy, and lymph node biopsy performed was suggestive of sarcoidosis. He was treated aggressively with intravenous steroids, but unfortunately continued to deteriorate rapidly. He was discharged to a rehab facility but quickly returned with worsening symptoms.





**Figure 3.** The HE stained section ( $\times 40$ ) reveals scattered glial cells with enlarged, pleomorphic nuclei with ground glass chromatin, typical of an active progressive multifocal leukoencephalopathy infection



**Figure 4.** This SV40 immunohistochemical stain ( $\times 40$ ) reveals the scattered pleomorphic glial cell nuclei to stain strongly due to a cross-reactivity among the polyoma viruses

Due to his continued decline, PML was considered as an alternative to neuro-sarcoidosis and stereotactic brain biopsy was performed. Pathology revealed extensive gliosis, abundant lipid-laden macrophages, and large ground glass viral inclusions [Figure 3]. SV40 was immunoreactive in the cells with viral cytopathologic changes confirming a diagnosis of progressive multifocal leukoencephalopathy [Figure 4]. The poor prognosis was discussed with the family and they decided to transition him to comfort care. The patient was discharged home with hospice and died in late September of 2017.

## DISCUSSION

The patient presented is a unique case of PML for three reasons. First, CSF testing was negative for JC Virus DNA. This caused a major delay in the diagnosis. Previous cases have been described where CSF PCR testing



by neutrophil pleocytosis or a mixed cellular response with neutrophils and lymphocytes. In later stages of tuberculous meningitis, lymphocytic inflammation predominates while neutrophils remain present until full clinical remission. The cytological picture of CNS viral infection predominantly shows a lymphocytic response. However, neutrophil pleocytosis may present in necrotizing cerebral processes of acute viral infections (e.g. herpes simplex virus-1 encephalitis or CNS vasculitis). Eosinophilic pleocytosis in the CSF is a hint at a parasitic infection, such as neurocysticercosis, a relatively common type of parasitic infection in North China. A cytological picture of eosinophil predominance in CSF, so called “eosinophilic meningitis”, indicates either a special type of neurocysticercosis (e.g. meningeal type of cysticercosis) or *Angiostrongylus cantonesis*, mostly in Southeastern China<sup>[7]</sup> [Figure 2].

The presence of plasma cells, the antibody-synthetic cells transformed from B-lymphocyte, in CSF indicates a chronic infection (e.g. tuberculosis, syphilis or borreliosis) or intrathecal humoral immunological response. Some infections lead to a higher content of atypical lymphoid or lymphoblastic cells in the CSF, which makes the differential diagnosis from lymphoma very difficult.

### CSF CYTOLOGY OF AUTOIMMUNE CNS DISEASE

Autoimmune CNS disorders include primary neuroimmune disorders [e.g. multiple sclerosis (MS), neuromyelitis optica spectrum disorders (NMOSD) and autoimmune encephalitis] and those secondary to systemic autoimmune disease.

Inflammatory reactions in CSF are observed in about half of AQP4-IgG positive NMOSD patients. The main inflammatory type is lymphocytic inflammation, while neutrophils and eosinophils are also seen in a few patients. Activated lymphocytes, activated mononuclear cells, and plasma cells can be detected in some patients' CSF. The positive rate of the specific oligoclonal bands in AQP4-IgG positive NMOSD is lower than that in multiple sclerosis. These CSF characteristics may contribute to the diagnosis of NMOSD. A mildly increased percentage of eosinophils is common in autoimmune CNS demyelination including NMOSD, acute disseminated encephalomyelitis and transverse myelitis. The demonstration of plasma cells in CSF of patients with MS is significantly correlated with pleocytosis and intrathecal IgG synthesis<sup>[8]</sup>.

Anti-NMDAR encephalitis is a major type of autoimmune encephalitis associated with autoantibodies against neuronal surface proteins. Intrathecal synthesis of autoantibodies is a pathological mechanism of this disease. CSF cytology findings include lymphocytic inflammation, occasionally with a mild increased percentage of neutrophils. Plasma cells are common in CSF of anti-NMDAR encephalitis which indicates intrathecal immunoglobulin synthesis<sup>[9]</sup>. For other type of autoimmune encephalitis without the mechanism of intrathecal immunoglobulin synthesis (e.g. anti-LGI1 encephalitis and anti-GABAbR encephalitis), the CSF cytology findings are normal or less reactive<sup>[10]</sup>.

Neuro-Behçet Syndrome (NBS) may present with various clinical manifestations. The most common neuroimaging finding is CNS midline structure involvement including brainstem, basal ganglion and thalamus. The CSF cytological findings, typically the presence of neutrophils, indicate the nature of neutrophilic vasculitis of NBS and are important clues in the diagnosis of NBS.

Neuropsychiatric systemic lupus erythematosus (SLE) is the CNS involvement of SLE. According to our observation, the CSF cytology was abnormal in 32.9% of SLE patients, which showed lymphocytic inflammatory reactions or lymphocyte - neutrophil inflammation. Among these cases with positive CSF cytological findings, activated lymphocytes, plasma cells and activated monocytes were frequently present. Abnormal endocytosis of monocytes, which presented as monocytes phagocytosing lymphocytes or plasma cells, was shown in some cases<sup>[11]</sup>.

**Table 1. CSF cytological syndrome with etiological indication**

CSF cytology	Clinical syndrome	Etiological consideration
Lymphatic inflammation	Acute encephalitis	Viral encephalitis Autoimmune encephalitis
Lymphatic inflammation	Acute mild or moderated meningism	Viral meningitis
Lymphatic inflammation	Shepherd with chronic meningitis or chronic encephalomyelitis	Neurobucellosis
Mixed reaction with lymphocyte and neutrophil	Recurrent brain-stem encephalitis or diencephalitis	Neuro-Behçet disease
Mixed reaction with lymphocytes and neutrophils	Chronic or subacute encephalitis	Tubercular meningitis Cryptococcal meningitis
Mixed reaction with Lymphocytes, eosinophils and plasma cells	Chronic meningitis	Neurocysticercosis
Eosinophilic inflammatory	Acute meningitis in patients from Southeastern China	Angiostrongylus cantonesis
Lymphatic inflammation with mild increased eosinophils	Acute encephalomyelitis or myelitis	Transverse myelitis ADEM NMOSD

CSF: cerebrospinal fluid; NMDAR: N-methyl-D-aspartate receptor; ADEM: acute disseminated encephalomyelitis; NMOSD: neuromyelitis optica spectrum disorder

CSF cytology findings in CNS inflammatory disease are relatively non-specific. However, when we consider the cytological findings under the clinical background of an individual patient, then the so-called “clinical-CSF cytological syndrome” can lead to a specific diagnosis [Table 1]. For example, in patients with recurrent CNS midline structure involvement and neutrophil pleocytosis or a mixed cellular response of CSF cytology, Neuro-Behçet disease should be highly suspected<sup>[12]</sup>.

## CSF CYTOLOGY OF NEOPLASTIC DISORDERS

Identification of tumor cells by CSF cytology is direct and specific evidence of leptomeningeal involvement by neoplasms. The appearance of malignant cells in CSF usually indicates generalized seeding of the leptomeninges by tumor cells. The prevalence of leptomeningeal involvement of different tumors is important to the cytologists and clinicians to make an accurate clinical-cytological conclusion. According to Prayson and Fischler<sup>[13]</sup>, the most commonly identified malignancy in CSF specimens in adults is metastatic neoplasms. Primary central nervous system neoplasms (e.g. medulloblastoma) account for a higher percentage of CSF specimens in the pediatric population than in the adult population. We reviewed CSF cytology results from PUMCH between 1984 and 2003, including 3922 specimens<sup>[14]</sup>. Forty-nine cases (1.25%) were positive for malignant cells. Diagnoses included metastatic tumors (26 cases), metastatic lymphoma/leukemia (7 cases), primary CNS neoplasms (10 cases) and malignant unclassified neoplasms (6 cases).

Cytology deals with single cells without histological architecture, so cytologists often face the challenge of arriving at a definitive final diagnosis. Immunocytochemistry and immunophenotyping by flow cytometry are helpful for CSF cytology<sup>[15]</sup>. For example, it was estimated that CSF cytology combined with immunocytochemistry could indicate diagnostic findings in 50% of cases with primary central nervous system lymphoma (PCNSL). Flow cytometric analysis (FCA) and polymerase chain reaction (PCR) of rearranged IgH and TCR genes of CSF are widely used in the diagnosis of PCNSL<sup>[16,17]</sup>. To study the diagnostic value of CSF cytology in the diagnosis of PCNSL, we retrospectively analyzed the data of 21 patients of PCNSL with positive CSF cytological findings<sup>[18]</sup>. Conventional CSF cytology, immunocytochemistry, flow cytometric analysis and PCR of rearranged *IgH* and *TCR* genes of CSF were performed. The clinical and neuroimaging types of 21 patients included meningeal type ( $n = 13$ ), parenchymal type ( $n = 4$ ), ependymal type ( $n = 3$ ), and optic type ( $n = 1$ ). The CSF of all the 21 patients had atypical lymphocytes, suggestive of lymphoma. Of the 20 cases in which immunocytochemistry was performed, 17 showed B-lymphocyte predominance, which was consistent with the diagnosis of B cell

lymphoma. FCA of 7 cases showed a significant increase in the percentage of B cells in 5 patients, indicating B cell lymphoma, and NK/T-lymphocyte predominance in 1 case, indicating an NK/T lymphoma. On analysis of the IgH and TCR genes in CSF of 4 patients, IgH monoclonal was found in 3 cases and TCR monoclonal in 1 case [Figure 2]. In fact, PCNSL is now one of the most common primary CNS neoplasms identified by comprehensive CSF cytological studies.

The application of molecular diagnostic techniques, including polymerase chain reaction and next-generation sequencing in CSF studies is booming in an era of precision medicine<sup>[19,20]</sup>. These novel techniques may be used to diagnose neoplastic meningopathy without cytological evidence<sup>[19,20]</sup>. However a final diagnosis based only on molecular techniques without morphological or cytological evidence should be avoided in clinical practice in order to prevent risks of over-diagnosis and misdiagnosis. CSF cytology is still the cornerstone of neurological diagnosis. The classical CSF examinations, including CSF cytology, still has their horizon in the era of precision neurology.

## DECLARATIONS

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Both authors drafted the manuscript, read and approved the final manuscript.

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There are no conflicts of interest.

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Not applicable.

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Not applicable.

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Review

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# The role of ubiquitinated TDP-43 in amyotrophic lateral sclerosis

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## Abstract

Deposition of intracellular ubiquitin inclusion in motor neurons is one of the leading pathogenic mechanisms of amyotrophic lateral sclerosis (ALS). The transactive response DNA binding protein-43 (TDP-43) is the main component of intracellular ubiquitin inclusion bodies in pathological deposits. TDP-43 is mainly distributed in the nucleus of neurons, and participates in nuclear RNA transcription, alternative splicing and mRNA stability regulation. The *tardbp*, as a coding gene, provides instructions for making TDP-43. After post-translational modification, the pathological TDP-43 induces pathological deposition in cells and is associated with neurodegenerative diseases, which is similar to tau in Alzheimer's disease and alpha-synuclein in Parkinson's disease. The pathogenic *tardbp* mutation can affect the localization of reverse transcription in the cell. This review summarizes the mechanisms underlying the pathogenesis of ALS by ubiquitination of TDP-43 protein.

**Keywords:** TDP-43 protein, ubiquitination, *tardbp*, amyotrophic lateral sclerosis

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a disease of progressive degeneration of motor neurons with an insidious onset. It is fatal due to progressive weakening of respiratory muscles. Lack of effective treatment has frustrated the medical community, and the underlying mechanism of ALS remains undetermined. Following the discovery of superoxide dismutase 1 (*SOD1*) mutation in familial ALS<sup>[1]</sup>, TAR DNA-binding protein 43 (*tdp43/tardbp*) inclusions have been found in ALS to be related to familial ALS<sup>[2]</sup> (fALS). Moreover, TDP-43 protein, as an intracellular ubiquitin inclusion, has also been identified in sporadic ALS patients<sup>[3,4]</sup>. The understanding of the pathogenic mechanism of ALS has been gradually changed by the



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discovery of *tdp43* mutation. In 2008, one observational study in France showed that 4% of familial ALS patients had a *tdp43* mutation<sup>[2]</sup>. It was also found in sporadic ALS patients, which bolstered knowledge of the pathological mechanism of ALS<sup>[2]</sup>. The abnormal intracellular ubiquitin inclusion was confirmed to be the cause of neuronal cell death<sup>[3,5]</sup>. Therefore, the highly ubiquitinated phosphorylated TDP-43 protein is closely related to the development of ALS. This review illustrates the development of TDP-43 in ALS and its underlying mechanism.

## NORMAL TDP-43 AND ITS FUNCTION

Normal TDP-43 is a protein with a length of 414 amino acids. It contains 2 RNA identity motifs (RRM1 and RRM2, respectively) at the N terminal<sup>[6,7]</sup> and hydrophobic sequence in the C-terminus<sup>[7]</sup>. The presence of the RRM2s is a distinguishing feature of heterogeneous nuclear ribonucleoprotein proteins (hnRNP) and, in general, these regions are known to mediate RNA recognition as well as protein-protein interactions. The first RRM (RRM-1) is necessary and sufficient to bind specific RNA or DNA sequences<sup>[8]</sup>. TDP-43 is encoded by *tardbp* which mainly distributed in the nucleus. Normal TDP-43 does not form inclusion bodies and is mainly involved in nuclear RNA transcription, mRNA precursor shear and mRNA stability regulation. Recruitment of full-length TDP-43 into cytoplasmic deposition formed inclusion bodies. Next, TDP-43 combines with RNA/DNA and regulates RNA metabolism at multiple levels, including transcription, RNA splicing, and mRNA stability. In TDP-43 knockout HeLa cells and HEK293 cell cultures, *tdp43* can regulate *add2* gene expression by increasing Add2 mRNA stability, which is closely related to synaptic aggregation, synaptic remodeling and stability<sup>[7,9]</sup>. UTRs at the 3' terminal of TDP-43 protein is a specific mRNAs that is associated with its distribution and aggregation in cells. TDP-43 takes charge of bidirectional transcription between the nucleus and cytoplasm. Its solubility and localization are closely related to its nuclear localization signal. In addition, it also affects the function of the protein<sup>[8]</sup>.

By monitoring the levels of TDP-43 oligomers *in vitro*, TDP-43 has been found to communicate through the axon in the cell by synaptic and microencapsulated uptake<sup>[10]</sup>. With the help of nuclear magnetic resonance, simulation and microscopy, a sub-region has been found cooperatively but transiently to be folded into an  $\alpha$ -Helical form that mediates TDP-43 phase separation<sup>[11]</sup>. It has also been illustrated that the low-complexity of TDP-43 in liquid-liquid and phase-separated *in vitro* granules demonstrates ALS-associated variants that disrupts interactions within the granules<sup>[11]</sup>. It is stabilized by extending its helices-structure and promotes binding between the molecules<sup>[11]</sup>. Stable helical conformation was adopted by residues Pro320-Leu340 in an isolated peptide of Met311-Gln360, while it is indeed lacking of any stable secondary structure. An observation at the glycine rich region of C-terminal domain is relatively conservative and related to the concentration and separation of molecules<sup>[12]</sup>. The concentration of TDP-43 molecules depends on its  $\alpha$ -Helical structure of the protein C-terminal fractures (CTF)<sup>[11]</sup>.

TDP-43 belongs to the family of the hnRNPs and is highly conserved among metazoans in both sequence and function<sup>[12]</sup>. In human cells, overexpression of TDP-43 decreases exon 9 recognition and specifically causes exon skipping *in vitro*<sup>[13]</sup>.

In relation to its function, TDP-43 has a variety of diverse roles including gene transcription, RNA splicing, RNA shuttling and translation, and microRNA biogenesis<sup>[14]</sup>. TDP-43 regulates various processes of transcription through RNA and DNA binding. Moreover, recent reports have shown that the protein interacts with the 3-UTRs of specific mRNAs<sup>[15]</sup>. In a previous study, the depletion of TDP-43 through RNA interference removes splicing inhibition caused by unfavorable (UG)mU(n) sequences, indicating that TDP-43 exerts a potent inhibitory effect *in vivo*<sup>[15]</sup>. More recently, new evidence that TDP-43 protein continuously shuttles between the nucleus and cytoplasm in a transcription-dependent manner has been reported<sup>[16]</sup>. The functional TDP-43 plays a dominant role in exon 9 splicing regulatory elements and shows that TDP-43 related splice site has determined the evolution of positive splicing regulatory elements



in contrast to this inhibition. On the other hand, TDP 43 was also found to repress cryptic exon splicing in order to promote cell survival<sup>[14]</sup>. TDP-43-dependent splicing defects, revealing TDP-43 extensively regulated cryptic splicing, are a significant overlap in genes that undergo TDP-43-dependent cryptic splicing repression<sup>[14]</sup>.

### THE ORIGIN OF PATHOLOGICAL TDP-43

Pathological TDP-43 mediated neuronal death is mainly caused by neurotoxicity and loss of TDP-43 function<sup>[17]</sup>. Phosphorylation and ubiquitination of TDP-43, the major features of pathological TDP-43, have not been detected in the normal brain. Phosphorylated and/or ubiquitinated TDP-43 have been found in the brain and spinal cord of patients with ALS<sup>[4]</sup>. In human studies, not all of TDP-43 inclusion bodies have been ubiquitinated, especially in the early stage of ALS, which suggests that ubiquitination is an advanced metabolic phenomenon in ALS disease<sup>[18]</sup>. The phosphorylated TDP-43 exists more commonly with serine (Ser) 379, Ser 403 + Ser 404, Ser 409 +, and Ser 410, which mainly between Ser 409-410. Most of the inclusion bodies of TDP-43 and TDP-43 25-kDa, as the degradation fragments of TDP 43, are detected in the phosphorylated form<sup>[4]</sup>. Therefore, the process of phosphorylation is likely preceded by ubiquitination. However, it is still unclear which form plays a more determinant role in mediating TDP-43 induced neurodegeneration.

In pathological conditions, TDP-43 protein degrades into two degradation fragments at the C-terminal<sup>[19]</sup>. By selectively expressing mutations in neurons and glial cells, the pathological TDP-43 protein is more commonly found to concentrate in neuronal cells, which may cause it failure to regulate synaptic plasticity and neuronal death<sup>[8,20]</sup>. Thus, mistakenly accumulated TDP-43 in motor neurons might be the initial mechanism of ALS onset<sup>[20]</sup>.

The fragments of TDP-43 protein can induce TDP-43 deposition, which is related to the ubiquitin proteasome system<sup>[5,21]</sup>. The overexpression of the full-length protein of TDP-43 and its aggregation can be detected in high expression of *tardbp* CTFs cells. Although both TDP-43 and TDP-43 fragments would be affected by the ubiquitin proteasome system, CTFs fragments are more likely to foster transcription without stopping due to the absence of two nuclear localization signals. In addition, Cdc48TS, as an enhancer of neurotoxicity, promotes the deposition of pathological TDP-43<sup>[22]</sup>. Thus, any influence on the ubiquitin proteasome system may increase the expression of ubiquitination of TDP-43.

### PATHOGENESIS OF TDP-43

In *Drosophila* motor neurons, the high expression of TDP-43 caused axonal swelling and impaired mobility. Moreover, impairment was more severe in the motor neurons of A315T mutant phenotype<sup>[23]</sup>. The TDP-43 peptide segments can form *in vitro* in both the wild-type and A315T mutant, which are misfolded like precipitation as seen using an electron microscope<sup>[23]</sup>. Therefore, the main pathogenesis of ALS may be caused by the abnormal accumulation of TDP-43 in motor neurons and secondary atrophy of neurons or glial cells.

According to the findings in polymerization kinetics study, the pathological C terminus had a prion-like domain structure. This prion domain is present in most of the pathogenic mutations of TDP-43<sup>[23,24]</sup>. The pathological C terminus is intrinsically disordered only with some nascent secondary structures in aqueous solutions, but processes the capacity to assemble into dynamic oligomers rich in  $\beta$ -sheet structures. These structures interact with nucleic acid, which triggers rapid aggregation for most mutants<sup>[25]</sup>. Although RNA-binding protein prion-like domains have no homology or sequence similarity to the human prion protein that forms infectious protein aggregates in new variant Creutzfeldt-Jakob disease, many of these proteins have been identified as the major components of cytoplasmic inclusions associated with subtypes of ALS and

frontotemporal dementia (FTD). In addition, the pathogenic mutations caused by *tardbp* are concentrated in the glycine enriched region of the C terminus<sup>[23,26]</sup>. So far, over 60 mutations in *tardbp* have been found to cause fALS and FTD<sup>[24]</sup>; they are listed in Table 1 as extracted from the ClinVar database. *Tardbp* mutations in the nucleus might disrupt the formation of alpha helices, or their ability to stabilize<sup>[11]</sup>. Mutations in the spiral region affect molecular binding, concentration and the separation phase. The aggregation of pathological TDP-43 is due to the overexpression and stacking of TDP-43 proteins. The TDP-43 prion-like domain appears to have an energy landscape, which allows the assembly of the wild-type sequence into dynamic oligomers only under very limited conditions. ALS-causing point mutations are sufficient to remodel it into a more favorable formation of amyloid and its irreversible aggregation, thus supporting the emerging view that such pathologic aggregation may occur via the exaggeration of functionally important assemblies<sup>[24]</sup>.

TDP-43 oligomers may further delay the release from each other<sup>[11]</sup>, resulting in the TDP-43 oligomerization in the nucleus, which is a possible mechanism of disruption of TDP-43<sup>[24]</sup>. Aging or inhibition of protein degradation may increase the toxicity of TDP-43 in glial cells and cause neuropathological changes.

TDP-43 C-terminus encodes a prion-like domain, widely presented in RNA-binding proteins like a prion-like domain. C-terminus is essential for solubility and cellular localization, because its deletion results in the formation of large nuclear and cytoplasmic aggregates<sup>[14]</sup>. Disruption of the RNA-recognition domain required for RNA and DNA binding, however, alters nuclear distribution by decreasing TDP-43 presence in the nucleoplasm.

The assembly of the wild-type sequence into dynamic oligomers was only seen under very limited conditions; ALS-causing point mutations are sufficient to remodel it to favor the amyloid formation or irreversible aggregation, thus supporting the emerging view that pathologic aggregation may occur via the exaggeration of functionally important assemblies<sup>[24]</sup>. Furthermore, the coupled capacity of TDP-43 in aggregation and membrane interaction may critically account for its high neurotoxicity<sup>[27]</sup>.

In addition, the proteinopathy of D169G and K263E mutants at the RRM domain of TDP-43 could form the basis of ALS, including the increased solvent-accessible surface area, conformational flexibility as well as unfolding of TDP-43, and the altered RNA conformation in TDP-43-RNA complex. These changes also brought the enhanced aggregation propensity in the cytoplasm<sup>[28]</sup>. These novel findings were important to illustrate the mechanism in the structural and functional aspects of ALS development.

## REDISTRIBUTION OF INTRACELLULAR TDP-43

The abnormal TDP-43 fragments would be re-distributed in the extracellular region of the nucleus<sup>[6]</sup>. More studies suggested that TDP-43 solubility and localization are particularly sensitive to disruptions that extend beyond the newly found nuclear localization signal and depend on a combination of factors that are closely connected to the functional properties of this protein<sup>[14]</sup>. TDP-43 fragmentation accelerates the formation of inclusion body and cell mRNA processing. The N-terminus fragment is highly distinctive, which promotes aggregation of the C-terminus structure<sup>[6]</sup>. When overexpression of TDP-43 and its C-terminal fragments in HEK293T cells, fragments of TDP-43 protein and TDP35 are recruited and removed into the cytoplasmic inclusion bodies<sup>[8]</sup>. TDP-35 participates in the aggregation of mRNA precursors, which makes the transformation of proteins into polymers easier. The insoluble fraction of ALS acts as a seed of TDP-43 aggregation when it is introduced in SH-SY5Y cells, and subsequently transmitted to other co-cultured cells<sup>[19,29]</sup>. Such extracellular accumulation, in a potentially more harmful way, is similar to the prion infections<sup>[30,31]</sup>. In addition, normal TDP-43 distribution in nucleus is not toxic to the cell, while only *tardbp* mutants cause redistribution in the extracellular region of the nucleus with neurotoxicity<sup>[3]</sup>. In a clinical study, previous work showed that accumulation of pathological TDP-43 or FUS coexist with

**Table 1. List of all TARDBP related mutations in ALS condition**

Name	Gene(s)	Condition(s)	Clinical significance	Variation ID	Allele ID
NM_007375.3(TARDBP):c.-126G>T	TARDBP	FTD, ALS, dominant	Uncertain significance	291727	275977
NM_007375.3(TARDBP):c.-122G>A	TARDBP	FTD	Benign	291728	275978
NM_007375.3(TARDBP):c.-117G>A	TARDBP	FTD, ALS, dominant	Uncertain significance	291729	275982
NM_007375.3(TARDBP):c.-110C>T	TARDBP	FTD, ALS, dominant	Uncertain significance	291730	275839
NM_007375.3(TARDBP):c.-77G>A	TARDBP	FTD, ALS, dominant	Uncertain significance	291731	275812
NM_007375.3(TARDBP):c.-42C>T	TARDBP	FTD, ALS, dominant	Uncertain significance	291732	275983
NM_007375.3(TARDBP):c.-12-10_-12-9delTT	TARDBP	FTD, ALS, dominant	Likely benign	291733	275813
NM_007375.3(TARDBP):c.87C>T (p.Ser29=)	TARDBP	Not provided	Uncertain significance	444152	437792
NM_007375.3(TARDBP):c.198T>C (p.Ala66=)	TARDBP	FTD not specified/ALS, dominant	Benign/likely benign	291734	275840
NM_007375.3(TARDBP):c.238+9C>T	TARDBP	FTD, ALS, dominant	Likely benign	291735	275850
NM_007375.3(TARDBP):c.239-15G>A	TARDBP	FTD, ALS, dominant	Uncertain significance	291736	275814
NM_007375.3(TARDBP):c.499A>G (p.Met167Val)	TARDBP	FTD, ALS, dominant	Uncertain significance	291737	275988
NM_007375.3(TARDBP):c.506A>G (p.Asp169Gly)	TARDBP	ALS type 10	Conflicting interpretations of pathogenicity	5233	20272
NM_007375.3(TARDBP):c.675A>G (p.Pro225=)	TARDBP	FTD, ALS, dominant	Likely benign	291738	275815
NM_007375.3(TARDBP):c.720G>A (p.Ala240=)	TARDBP	FTD, ALS, dominant	Uncertain significance	291739	275851
NM_007375.3(TARDBP):c.859G>A (p.Gly287Ser)	TARDBP	ALS type 10/motor neuron disease	Conflicting interpretations of pathogenicity	21483	34335
NM_007375.3(TARDBP):c.869G>C (p.Gly290Ala)	TARDBP	ALS type 10	Pathogenic	5231	20270
NM_007375.3(TARDBP):c.881G>T (p.Gly294Val)	TARDBP	ALS type 10	Pathogenic	21484	34336
NM_007375.3(TARDBP):c.881G>C (p.Gly294Ala)	TARDBP	ALS type 10	Pathogenic	5230	20269
NM_007375.3(TARDBP):c.883G>A (p.Gly295Ser)	TARDBP	ALS type 10	Pathogenic	21485	34337
NM_007375.3(TARDBP):c.892G>A (p.Gly298Ser)	TARDBP	ALS type 10	Pathogenic	5232	20271
NM_007375.3(TARDBP):c.943G>A (p.Ala315Thr)	TARDBP	ALS type 10	Pathogenic	5236	20275
NM_007375.3(TARDBP):c.945G>A (p.Ala315=)	TARDBP	Not provided	Uncertain significance	374720	361606
NM_007375.3(TARDBP):c.991C>A (p.Gln331Lys)	TARDBP	ALS type 10	Pathogenic	5229	20268
NM_007375.3(TARDBP):c.1009A>G (p.Met337Val)	TARDBP	ALS type 10	Pathogenic	5228	20267
NM_007375.3(TARDBP):c.1028A>G (p.Gln343Arg)	TARDBP	ALS type 10	Pathogenic	5235	20274
NM_007375.3(TARDBP):c.1042G>T (p.Gly348Cys)	TARDBP	ALS type 10/not provided	Pathogenic	5234	20273
NM_007375.3(TARDBP):c.1043G>T (p.Gly348Val)	TARDBP	Motor neuron disease	Pathogenic	266064	260865
NM_007375.3(TARDBP):c.1098C>G (p.Ala366=)	TARDBP	FTD not specified/ALS, Dominant	Benign/likely benign	291740	275852
NM_007375.3(TARDBP):c.1122T>G (p.Tyr374Ter)	TARDBP	Motor neuron disease	Uncertain significance	266065	260866
NM_007375.3(TARDBP):c.1144G>A (p.Ala382Thr)	TARDBP	ALS type 10/FTD with TDP43 inclusions, TARDBP-related/ not provided	Pathogenic/likely pathogenic	21474	34326
NM_007375.3(TARDBP):c.1150G>C (p.Gly384Arg)	TARDBP	ALS type 10	Pathogenic	190399	188225
NM_007375.3(TARDBP):c.1153T>G (p.Trp385Gly)	TARDBP	ALS type 10	Pathogenic	190400	188226
NM_007375.3(TARDBP):c.*83T>C	TARDBP	ALS type 10	Pathogenic	21465	34317
NM_007375.3(TARDBP):c.*129T>C	TARDBP	FTD, ALS, dominant	Uncertain significance	291741	276082
NM_007375.3(TARDBP):c.*159A>C	TARDBP	FTD, ALS, dominant	Uncertain significance	291742	275989

NM_007375.3( <i>TARDBP</i> ):c.*208G>A	<i>TARDBP</i>	FTD, ALS, dominant	Likely benign	291743	275853
NM_007375.3( <i>TARDBP</i> ):c.*214T>C	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291744	275816
NM_007375.3( <i>TARDBP</i> ):c.*505delA	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291745	275994
NM_007375.3( <i>TARDBP</i> ):c.*666G>A	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291746	275995
NM_007375.3( <i>TARDBP</i> ):c.*697G>A	<i>TARDBP</i>	ALS type 10/FTD with TDP43 inclusions, <i>TARDBP</i> -related	Pathogenic	5239	20278
NM_007375.3( <i>TARDBP</i> ):c.*842G>A	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291747	275996
NM_007375.3( <i>TARDBP</i> ):c.*862G>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291748	275855
NM_007375.3( <i>TARDBP</i> ):c.*963C>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291749	275856
NM_007375.3( <i>TARDBP</i> ):c.*1008T>G	<i>TARDBP</i>	FTD, ALS, dominant	Likely benign	291750	275998
NM_007375.3( <i>TARDBP</i> ):c.*1081C>T	<i>TARDBP</i>	FTD, ALS, dominant	Likely benign	291751	276021
NM_007375.3( <i>TARDBP</i> ):c.*1084A>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291752	276022
NM_007375.3( <i>TARDBP</i> ):c.*1597_*1600delTGTT	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291753	275859
NM_007375.3( <i>TARDBP</i> ):c.*1622A>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291754	275860
NM_007375.3( <i>TARDBP</i> ):c.*1623T>A	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291756	276025
NM_007375.3( <i>TARDBP</i> ):c.*1633delT	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291755	275867
NM_007375.3( <i>TARDBP</i> ):c.*1795A>G	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291757	275817
NM_007375.3( <i>TARDBP</i> ):c.*2005T>C	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291758	276105
NM_007375.3( <i>TARDBP</i> ):c.*2029C>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291759	275868
NM_007375.3( <i>TARDBP</i> ):c.*2046T>G	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291760	276026
NM_007375.3( <i>TARDBP</i> ):c.*2154G>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291761	276108
NM_007375.3( <i>TARDBP</i> ):c.*2252A>G	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291762	275872
NM_007375.3( <i>TARDBP</i> ):c.*2294_*2295insGTTT	<i>MASP2</i>   <i>TARDBP</i>	FTD, <i>MASP2</i> deficiency/ALS, dominant	Benign	291763	276114
NM_007375.3( <i>TARDBP</i> ):c.*2331A>G	<i>MASP2</i>   <i>TARDBP</i>	FTD, <i>MASP2</i> deficiency/ALS, dominant	Likely benign	291764	275874
NM_007375.3( <i>TARDBP</i> ):c.*2334G>A	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291765	276127
NM_007375.3( <i>TARDBP</i> ):c.*2360C>T	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291766	276128
NM_007375.3( <i>TARDBP</i> ):c.*2538delC	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291767	275875
NM_007375.3( <i>TARDBP</i> ):c.*2740G>A	<i>TARDBP</i>	FTD, ALS, dominant	Likely benign	291768	276138
NM_007375.3( <i>TARDBP</i> ):c.*2750G>A	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291769	275827
NM_007375.3( <i>TARDBP</i> ):c.*2773A>G	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291770	276140
NM_007375.3( <i>TARDBP</i> ):c.*2829dupT	<i>TARDBP</i>	FTD, ALS, dominant	Uncertain significance	291771	275835
NM_006610.3( <i>MASP2</i> ):c.*225T>C	<i>MASP2</i>   <i>TARDBP</i>	FTD, <i>MASP2</i> deficiency/ALS, dominant	Benign	291772	275885
NG_008734.1:g.19080G>A	<i>MASP2</i>   <i>TARDBP</i>	FTD, ALS, dominant	Likely benign	368798	353027
NM_006610.3( <i>MASP2</i> ):c.1617T>C (p.Asn539=)	<i>MASP2</i>   <i>TARDBP</i>	FTD, <i>MASP2</i> deficiency/ALS, dominant	Likely benign	291779	275924

FTD: frontotemporal dementia; ALS: amyotrophic lateral sclerosis; *MASP2*: Mannan-binding lectin-associated serine protease-2

misfolded HuWtSOD1 in patient motor neurons, and can trigger its misfolding in cultured cells<sup>[31]</sup>. *In vitro*, immunocytochemistry and immunoprecipitation were used to demonstrate that TDP-43 or FUS-induced misfolded HuWtSOD1 can propagate from cell-to-cell via conditioned media, and seed cytotoxic misfolding of endogenous HuWtSOD1 in the recipient cells in a prion-like fashion<sup>[31]</sup>. While siRNA in recipient cells and incubation of conditioned media with misfolded SOD1-specific antibodies could inhibit intercellular transmission *in vitro*<sup>[30]</sup>, intercellular spread of SOD1 misfolding is not accompanied by transmission of TDP-43 or FUS pathology.

### The spreading of pathological TDP-43

*In vivo*, TDP-43 protein is found in secreted exosomes from Neuro2a cells and primary neurons but not astrocytes and microglia<sup>[33]</sup>. The pathological TDP-43 protein aggregation and autophagy inhibition promote exosomal secretion of TDP-43<sup>[29]</sup>. The levels of exosomal full length TDP-43 and C-terminal fragment species are upregulated in the brain of ALS patients. If Neuro2a cells are exposed to the cerebrospinal fluid of ALS patients, the deposition of intracellular pathological TDP-43 proteins would be take place. In addition, Neuro2a cells can have TDP-43 deposition by regulating silent genes or inhibiting exocrine secretion. Upregulation of exocrine secretion in the TDP-43<sup>A315T</sup> mutant transgenic mice exacerbates the disease process. Exosome secretion is considered a key pathway for clearance of pathological TDP-43<sup>[29]</sup>.

To study the potential propagation of TDP-43, a HEK293 cell culture model was used, which supports the propagated misfolding of HuWtSOD1. It showed significant protein expression in cells transfected with TDP-43 constructs, but no expression in the incubated cells, indicating that the conditioned media contains no active residual lipofectamine reagent and that the transfection-encoded TDP-43 protein does not transmit to recipient HEK293 cell cultures<sup>[31]</sup>. Thus, pathological TDP-43 is cleared via the proteasome, which reduces efficient clearance of mis-folded SOD1.

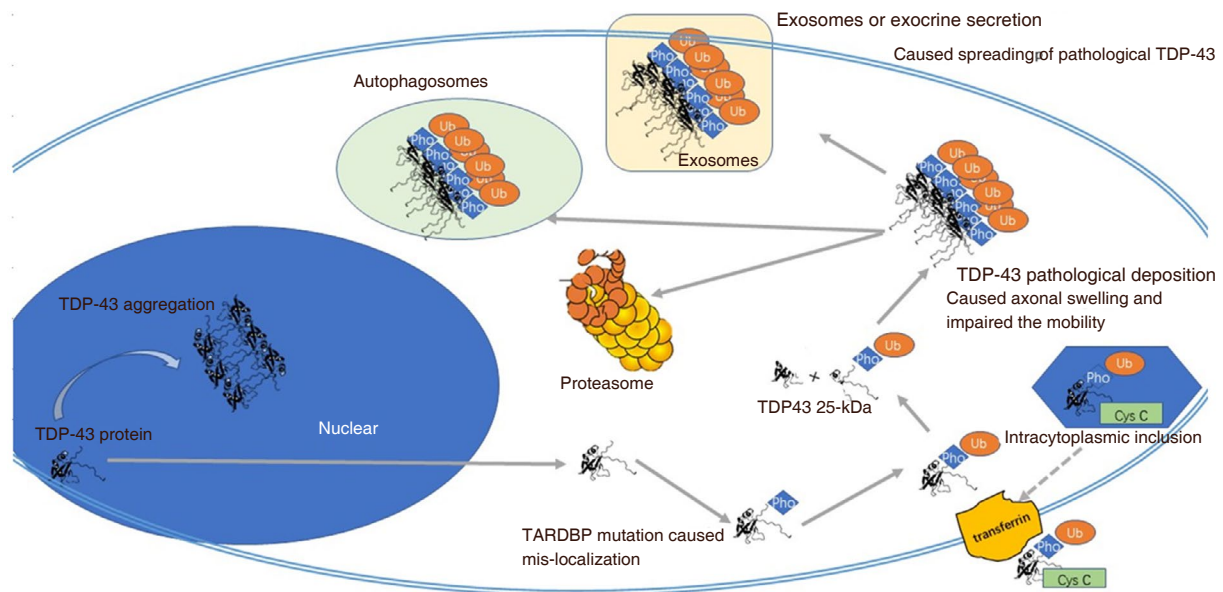
Recently, novel intracytoplasmic inclusions immunoreactive for phosphorylated transactivation response TDP-43 (p-TDP 43) were found in anterior horn cells in a case of a sporadic amyotrophic lateral sclerosis (sALS) patient. His spinal cord showed severe degeneration involving the anterior and lateral funiculi, whereas the posterior funiculus was preserved. Most neurons in the anterior horn and Clarke's column were markedly lost, while some remaining anterior horn cells had round and densely eosinophilic or amphophilic intracytoplasmic inclusions<sup>[32]</sup>. They were immune-reactive for ubiquitin, p-TDP-43, cystatin C and transferrin<sup>[32]</sup>. On confocal laser microscopy, cystatin C was found to consistently surround p-TDP-43 within the inclusions. It was a key finding that these unique inclusions may have been formed under a specific condition whereby p-TDP-43 and cystatin C interacted with each other<sup>[33]</sup>.

### Removal of pathological TDP-43

Although the *tardbp* gene mutation is uncommon, other gene mutations related to ALS can also lead to abnormal intracellular TDP-43 levels<sup>[29,34]</sup>. Therefore, it is necessary to analyze the clearing pathway of pathological TDP-43. The published study has reported such process not only involved the dynamic transportation by exocrine secretion and small vesicles, but also by autophagy as well<sup>[26]</sup>. With overcoming the confounding effects of aggregation and toxicity, pathogenic mutations that significantly shorten TDP-43 half-life were found in a single-cell optical method. A novel autophagic flux assay combined with an *in silico* screen identified compounds that effectively stimulate autophagy in neurons though enhancement of TDP-43 clearance and reduction of its mis-localization. On the other hand, the induction of autophagy can improve scavenging ability of TDP-43, enhancing the primary neuronal survival capacity<sup>[26]</sup>. TDP-43 causes differential pathology in neuronal versus glial cells in the mouse brain by increasing the TDP-43 clearance which can slow down the progression of ALS<sup>[26]</sup>.

The accumulation of intracellular TDP-43 is also related to the impairment of TDP-43 CTFs fragment clearance. The autophagy mediated TDP-43 CTFs fragmentation is caused by the failure of the phagocytosis





**Figure 1.** The role of ubiquitinated tdp43 that forms aggregates in amyotrophic lateral sclerosis

of membrane vesicles<sup>[26]</sup>, which suggested that autophagy also affected the transcription capacity of TDP-43 proteins. In addition, several studies have illustrated that TDP-43 concentration may increase toxicity in HeLa cells, suggesting that the autophagy system and the ubiquitin proteasome system may affect transcription of TDP-43<sup>[34]</sup>.

TDP-43 mitochondrial localization inhibitory peptide can also abolish cytoplasmic TDP-43 accumulation, restore mitochondrial function, prevent neuronal loss, and alleviate motor-coordinative and cognitive deficits in adult hemizygous TDP-43<sup>M337V</sup> mice<sup>[35]</sup>.

## TARGETING TDP-43 AS A POTENTIAL TREATMENT FOR ALS

Due to the fact that ALS patients demonstrate the inability of the cell's protein garbage disposal system to "pull out" and destroy TDP-43, a therapy targeting TDP-43 removal shows promise in clinical treatment. In a pilot study, researchers delivered parkin genes to neurons which slowed down ALS pathologies linked to TDP-43<sup>[36]</sup>. In another animal model, increased expression of UPF1, the master regulator of a nonsense-mediated decay pathway, can significantly protect mammalian motor neurons from TDP-43 mediated toxicity. UPF1 has shown promising results in animal models of ALS involving TDP-43 dysfunction and provides a rationale for developing gene-based therapies for ALS indicating the efficacy of a UPF1-based therapy in animal models of TDP-43 induced ALS pioneered in this laboratory<sup>[37]</sup>. Similarly, overexpression of the mammalian Sisl homologue, DNAJB1, relieves TDP-43 mediated toxicity in primary rodent cortical neurons, suggesting that Sisl and its homologues may have neuroprotective effects in ALS<sup>[38]</sup>.

In ALS disease progression, TDP-43 is ubiquitinated, hyper-phosphorylated, and cleaved to form intranuclear and cytosolic aggregates. There is an overall shift in its localization from the nucleus to the cytoplasm and axons [Figure 1]. Over 60 dominant missense mutations have been defined in TDP-43, which may have an increased propensity to cleavage and may be resistant to degradation. More stimulation studies in this mechanism show that TDP-43 antibodies could be one potential strategy for disease intervention.

In summary, the pathogenic mechanism of ubiquitinated TDP-43 in ALS, including the origin and redistribution of pathological TDP-43, has been studied intensively in the past ten years. Currently,



phosphorylation and ubiquitination of TDP-43 have been identified and recognized to be the source of pathological protein aggregation, inclusion bodies formation and abnormal exosome secretion. Similar to prion propagation and autophagy, these findings may help understand the relationship between ubiquitinated TDP-43 and ALS pathogenesis. More research is needed on the metabolic pathways of neurotoxic TDP-43 fragments.

## DECLARATIONS

### Authors' contributions

Designed this study: Dong Y, Chen Y

Participated in material review and draft the manuscript: Dong Y

Revised the manuscript: Chen Y

### Financial support and sponsorship

None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Case Report

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# Hypodysfibrinogenemia in a young patient with recurrent strokes

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## Abstract

Stroke is a complex disease, but in some instances is the direct result of a monogenic disease. Here we report the case of a 44-year-old Italian man who experienced recurrent transitory ischemic attacks and strokes. He also had right fetal-type posterior cerebral artery. He was diagnosed with congenital hypodysfibrinogenemia due to a mutation leading to a truncated fibrinogen gamma chain. Further studies are needed to better elucidate the links between fibrinogen dysfunction and stroke. Hypodysfibrinogenemia should be included among the monogenic diseases associated with ischemic stroke. Physicians should be aware of this condition, which may be detectable on routine assays.

**Keywords:** Coagulation, dysfibrinogenemia, fibrinogen, genetics, ischemic stroke, monogenic

## INTRODUCTION

Stroke is a complex disease resulting from the interplay of genetics and environment. In some instances stroke is the direct result of a monogenic disease, mainly in young adults. Here we report the case of a 44-year-old Italian man who experienced recurrent transitory ischemic attacks (TIAs) and strokes from the age of 38 years. At last, he was diagnosed with congenital hypodysfibrinogenemia due to a mutation leading to a severely truncated fibrinogen gamma chain.

## CASE REPORT

Our patient presented at 38 years with recurrent TIAs (~1/month), characterized by sensory disturbances



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on the left side of the body. Both the family history and past medical history were unremarkable. At that time, brain magnetic resonance imaging (MRI) was normal except few non-specific white matter lesions. Electroencephalography (EEG) (including prolonged monitoring) was normal. He was prescribed acetylsalicylic acid (ASA) 100 mg/day, but chose to discontinue using after an episode of nosebleeding.

At age 40 years, he presented with left hemiparesis due to a right mesial frontal ischemic stroke [Figure 1A and B]. The patient, who was prescribed with ASA 300 mg/day, gradually recovered until the following year (aged 41 years), when he was examined because of acute-onset left hemianopia. MRI showed an acute ischemic lesion in the right temporo-parietal-occipital regions [Figure 1C and D]. Ivy bleeding time was prolonged to 14 min (normal 2.5-11 min<sup>[1]</sup>) confirming appropriate antiaggregation.

Cerebrovascular angiography, performed under the hypothesis of a vasculitis, showed only a right fetal-type posterior cerebral artery (PCA) [Figure 1E and F]. Brain 18F-fluorodeoxyglucose positron emission tomography and cerebrospinal fluid examination were unremarkable. Complete cardiologic screening was normal. EEG showed mild epileptiform alterations on the right hemisphere. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), Fabry disease and mitochondrial disorders were excluded by appropriate biochemical and genetic studies.

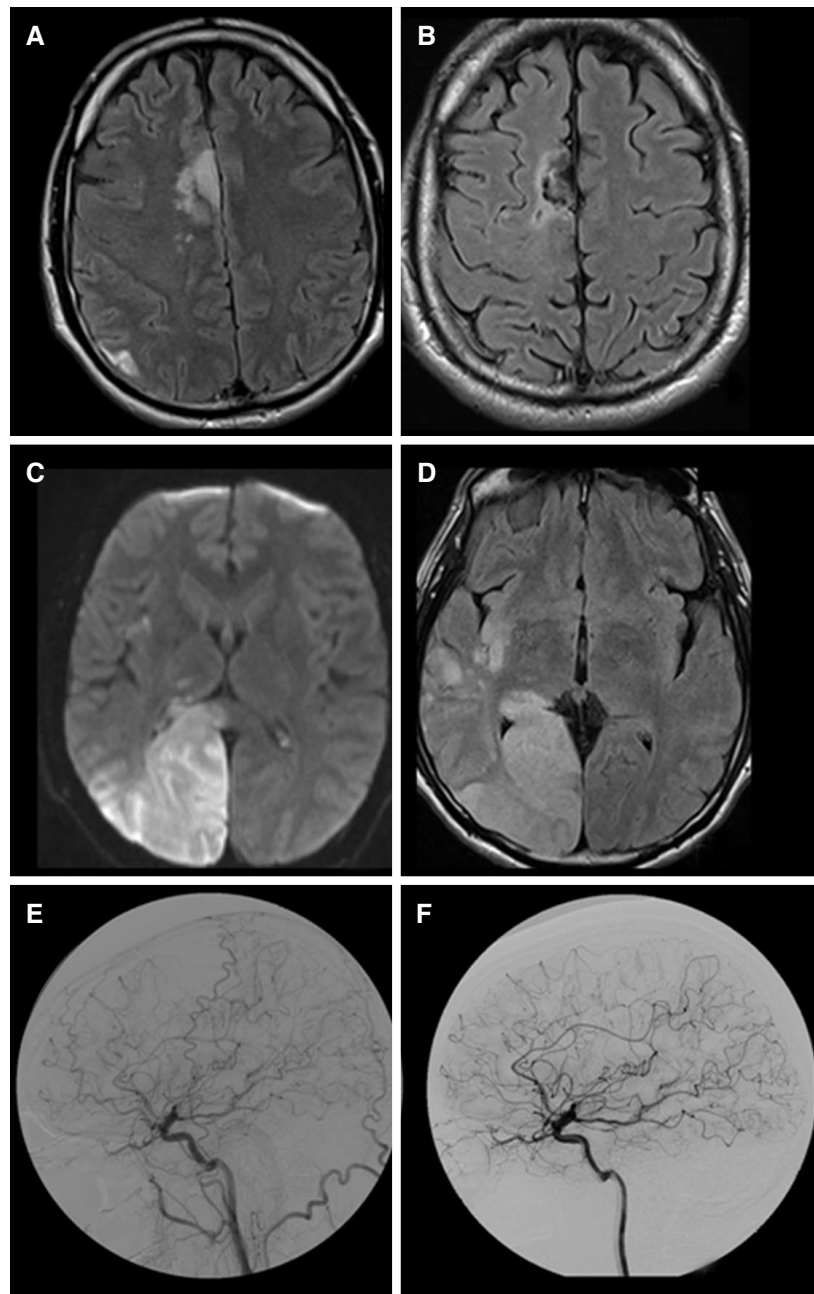
Routine laboratory assays (including D-dimer, prothrombin time, activated partial thromboplastin time, factor V, antithrombin, protein C, protein S, activated protein C resistance, lupus anticoagulans, anti-antinuclear antibodies, anti-smooth muscle antibodies, anti-cardiolipin antibodies, extractable nuclear antigen antibodies, anti-neutrophil cytoplasmic antibodies and antiphospholipid antibodies) were normal, except for a mild but long-lasting hypofibrinogenemia (115-180 mg/dL; normal 200-400 mg/dL).

Hypofibrinogenemia is most often caused by heterozygous mutations in one of the three fibrinogen chains, whereas the same homozygous mutations commonly lead to afibrinogenemia, a bleeding disease. Most cases of heterozygous disorders of fibrinogen affect quantity and quality of circulating fibrinogen (hypodysfibrinogenemia, OMIM #616004), and may lead to both bleeding diathesis and thromboembolic complications (even in the same patient). This predisposition for thrombosis has been ascribed to delayed plasmin digestion<sup>[2]</sup>.

Ischemic stroke was reported only in exceptional patients with homozygous gamma chain (FGG) mutations<sup>[3]</sup>, but large case-control association studies showed that polymorphisms in the genes encoding the alpha and gamma chains may lead to an increased risk of ischemic stroke, likely mediated by qualitative effects on fibrinogen (and fibrin)<sup>[4]</sup>.

These considerations prompted a genetic work-up in our patients. At last, sequencing of the three fibrinogen chains showed the 945C>T mutation in the *FGG* gene, leading to a stop codon (p.Arg108Stop of the mature chain). This mutation was previously reported in ten consanguineous, homozygous afibrinogenemic patients from Lebanon<sup>[5]</sup>. These patients had bleeding diathesis. Unfortunately, no detailed clinical data on the heterozygous relatives were provided. However they had, as our patients, mild hypofibrinogenemia with other coagulation tests in the normal range. Of note, expression studies demonstrated that this nonsense mutation affected neither mRNA splicing nor stability, but led to the production of an unstable, severely truncated fibrinogen gamma chain that is not incorporated into a functional fibrinogen hexamer (in this study the mutation was denoted as p.Arg134Stop, considering the immature chain)<sup>[5]</sup>.

The genetic finding confirmed the diagnosis of congenital hypodysfibrinogenemia in our patient, who in the 3 years following the 2nd stroke did not experience further TIAs or stroke (under double-antiaggregation therapy with ASA 100 mg/day and clopidogrel 75 mg/day). He had only 3 episodes of simple motor partial seizures successfully treated with oxcarbazepine.



**Figure 1.** Recurrent strokes and fetal-type PCA in our patient. (A, B) Right mesial frontal ischemic stroke at age 40 years, and its 6-month evolution (FLAIR axial MRI images); (C, D) acute ischemic lesion in the right temporo-parietal-occipital regions at age 41 years (diffusion-weighted and FLAIR axial MRI images); (E, F) two consecutive angiograms (at the time of the first stroke and after 6 months) showing the fetal-type right PCA, originating from the right internal carotid artery. PCA: posterior cerebral artery; MRI: magnetic resonance imaging

## DISCUSSION

To our knowledge, this is the first report of an heterozygous fibrinogen mutation in a young patient with recurrent TIAs and strokes. Further studies are needed to better elucidate the links between fibrinogen function (and dysfunction) and ischemic stroke. It is likely other genetic and/or environmental factors may have a role.

From this perspective, an intriguing observation is the lateralization of all the TIAs and strokes of our patient in the right hemisphere (in the presence of a right fetal-type PCA). Even if the association between

fetal PCA and ischemic stroke is debated<sup>[6]</sup>, fetal PCA may have some hemodynamic impact (e.g. faster perfusion transit times ipsilateral to fetal origin of the PCA)<sup>[7]</sup>, and a detrimental interaction of the two factors (hemodynamics and coagulation) cannot be ruled out.

In conclusion, hematologists, geneticists and neurologists should be aware of this condition, which in most cases may easily be detectable on routine assays, and hypodysfibrinogenemia should be included among the monogenic diseases<sup>[8]</sup> associated with ischemic stroke in younger adults.

## DECLARATIONS

### Authors' contributions

Prepared the manuscript: Orsucci D

Revised the manuscript: Mazzoni M

Clinically studied the patient: Salvetti S, Vista M

Performed the hematological examinations: Margelli M

Performed the angiographic studies: Puglioli M

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None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

The manuscript does not contain patient identifiable data.

### Ethics approval

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Case Report

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# Steroids and plasma exchange in Isaacs' syndrome with anti-Caspr2 antibodies

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## Abstract

Isaacs' syndrome is a disease characterized by nerve hyperexcitability. The patients are commonly treated with symptomatic therapies and immunomodulatory approaches, but no clinical trials are available to date. Here, we report the case of an anti-Caspr2-positive patient, presenting with continuous muscle twitches and diffuse muscle pain. He experienced a nearly complete clinical response to intravenous high-dose steroids combined with plasma exchange, sustained for at least 1 year. Our experience suggests that methylprednisolone 1000 mg/day × 5 days and consecutive tapering followed by plasma exchange may be efficient and well tolerated in patient with Isaacs' syndrome due to anti-Caspr2 antibodies.

**Keywords:** Contactin-associated protein-2, Isaac, neuromuscular hyperexcitability, neuromyotonia, voltage-gated potassium channel

## INTRODUCTION

Isaacs' syndrome ("acquired neuromyotonia") is a disease characterized by peripheral nerve hyperexcitability and spontaneous and continuous skeletal muscle overactivity presenting as twitching and painful cramps, often accompanied by stiffness, pseudomyotonia, pseudotetany and weakness<sup>[1]</sup>. The commonest acquired form is autoimmune, caused by antibodies against nerve voltage-gated potassium channels (VGKC). Patients are commonly treated with symptomatic therapies (carbamazepine, phenytoin, lamotrigine or valproate) and



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immunomodulatory approaches, but no clinical trial is available to date and the optimal treatment approach is unknown<sup>[1]</sup>. Here, we report the case of a patient with Isaacs' syndrome tested positive for anti-contactin-associated protein-2 (Caspr2) antibodies.

## CASE REPORT

A 74-year-old Italian man, with unremarkable family history and without any significant comorbidities, reported diffuse muscle pain for the last three weeks. In the previous year he had occasionally presented muscle cramps. The pain had gradually worsened so that required the use of morphine intravenously and carbamazepine (400 mg/day) and pregabalin (225 mg/day) oral administration. Neurological examination revealed continuous muscle twitches in upper and lower limbs and in the facial muscles [Video 1]. The remaining examination was unremarkable.

Routine laboratory testing showed increased creatine kinase (CK) levels (579 U/L; normal < 190), mild anemia (haemoglobin  $\approx$  10 g/dL), increased erythrocyte sedimentation rate (74 mm/h; normal < 40) and C-reactive protein levels (13.6 mg/dL; normal < 0.5). Serological studies were negative for HIV, syphilis and *Borrelia*. Autoantibodies associated with rheumatic diseases, acetylcholine receptor and MuSK antibodies, anti-gangliosides, anti-Hu/Yo/Ri/CV2/Ma2, anti-amphiphysin and anti-GAD antibodies were not detected in the patient's serum. Cerebrospinal fluid examination (CSF) showed moderately increased protein levels (73 mg/dL; reference range 15-40 mg/dL), without any signs of intrathecal immunoglobulin synthesis.

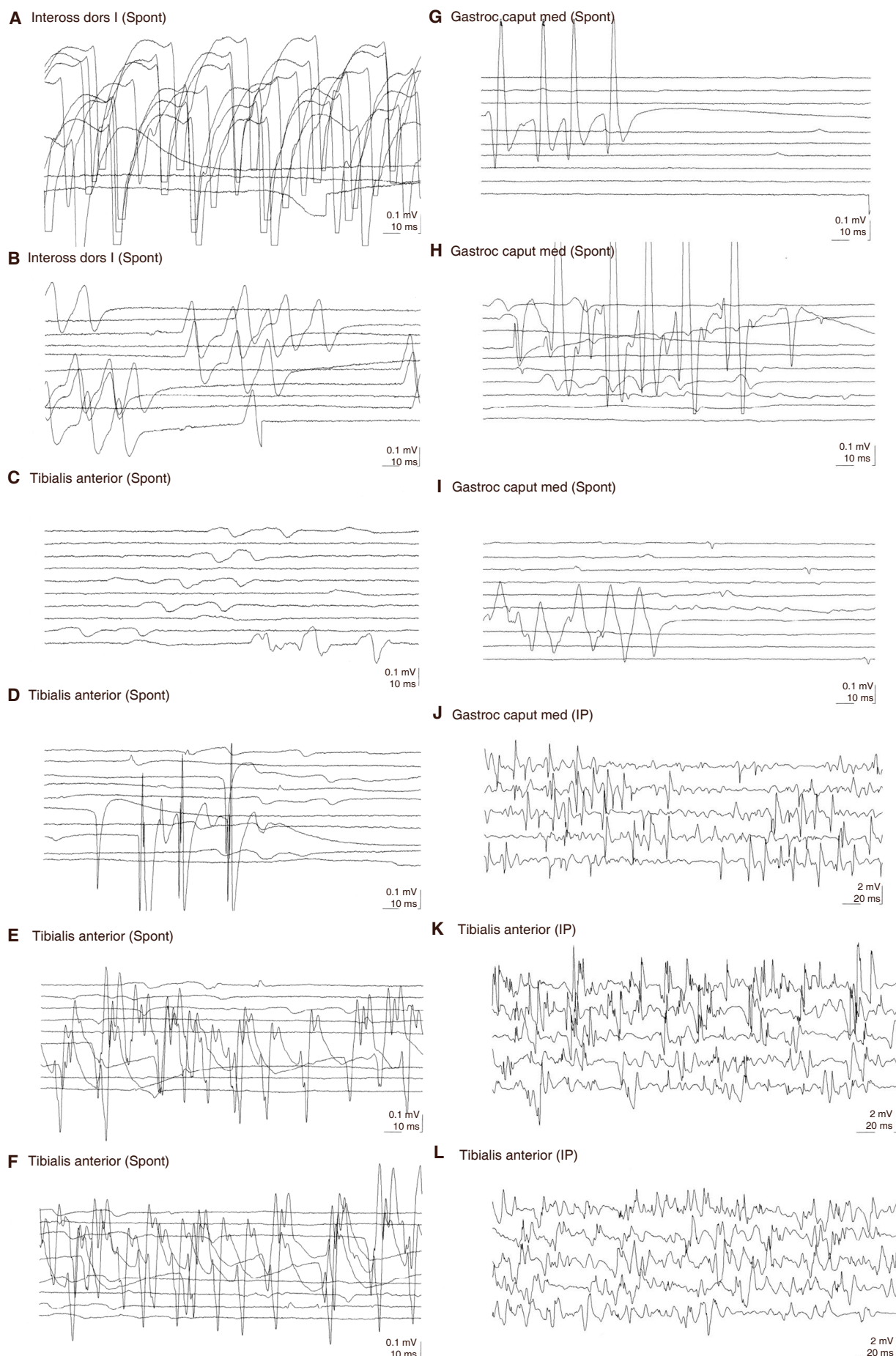
Total-spine and brain magnetic resonance imaging (MRI) and electroencephalography were normal. An extensive screening search for malignancies, including chest/abdomen computed tomography, gastroscopy, colonoscopy, and tumour markers measurement resulted negative, whereas a segmental Crohn's disease was diagnosed. Sensory and motor evoked potentials and nerve conduction studies were normal, whereas electromyography (EMG) showed continuous spontaneous activity in all the examined muscles [Figure 1], suggesting the diagnosis of Isaacs' syndrome. At last, this diagnosis was confirmed by the finding of anti-Caspr2 antibodies in the patient's serum.

Therefore, the patient was treated with high-dose corticosteroids (intravenous methylprednisolone 1000 mg/day  $\times$  5 days and consecutive tapering to prednisone 25 mg/day) followed by 5 sessions (about 2 h every other day) of plasma exchange (PEX), with immediate remission of the muscle twitches and pain at the end of the corticosteroid cycle and after the first PEX session [Video 2]. EMG confirmed this excellent improvement [Video 2]. At discharge, CK levels were normal. One year after discharge, the disease was still well controlled with low-dose oral corticosteroids (prednisone 25 mg/day) and symptomatic therapy with carbamazepine and pregabalin. Five years after discharge, the disease is excellently controlled by azathioprine 125 mg/day, which was prescribed as a corticosteroid-sparing treatment. Prednisone administration was interrupted. An extensive screening search for malignancies was repeated and was still normal.

## DISCUSSION

Neuromyotonia is a syndrome of spontaneously occurring muscle activity of peripheral nerve origin, which can be triggered by voluntary or induced muscle contraction<sup>[2]</sup>. It is one among several causes of visible myokymia. The abnormal activity is characterized electromyographically by doublet, triplet or multiplet single unit discharges that have a high intraburst frequency, the frequency of the bursts themselves being irregular<sup>[2]</sup>. Our patient fulfilled these EMG criteria [Figure 1].

Anti-VGKC autoantibodies have been identified in patients with acquired neuromyotonia, limbic encephalitis, or a combination of both (Morvan's syndrome). However, recent studies have shown that in fact



**Figure 1.** Continuous spontaneous activity in all the examined muscle districts. Electromyography showing abnormal repetitive spontaneous activity of the muscle fibers (doublet, triplet, or multiple single-unit discharges with high, irregular intraburst frequency) in all the muscle groups (reported in the figure). (A-E) Right side; (F-G) left side. Spont: spontaneous activity

the great majority of “anti-VGKC” autoantibodies are not directed against the potassium channel subunits but against three proteins that are complexed with these channels, including the cell-adhesion molecule Caspr2<sup>[3]</sup>. VGKC-antibodies define neurological conditions that are usually immunotherapy-responsive, but patients with anti-Caspr2 antibodies could have an increased risk of an underlying tumour and a poor prognosis<sup>[3]</sup>.

Caspr2 antibodies bind to the juxtaparanodal regions of myelinated fibers in brain and peripheral nerve<sup>[4]</sup>. Patients with anti-Caspr2 antibodies may show both peripheral and central nervous system features<sup>[3,4]</sup>. Neuropathic pain may be a significant and rather specific manifestation of anti-Caspr2 autoimmunity; hyperexcitability of nociceptive pathways has been implicated<sup>[5]</sup>. Peripheral neuromuscular hyperexcitability and pain were the main clinical features in our patient; no clinical or MRI signs of limbic encephalitis were noted. Additional work is required to explain different clinical phenotypes in patients with autoantibodies against Caspr2.

Isaacs' syndrome has been described in combination with other autoimmune disorders<sup>[6]</sup>, including myasthenia gravis<sup>[7]</sup>, or associated with a variety of neoplasms, including thymoma and lymphoma<sup>[8]</sup>. It can be diagnosed several years before a neoplasm is discovered<sup>[8]</sup>. In our case, an extensive screening was negative for neoplasms, but revealed a segmental Crohn's disease (to our knowledge, this is the first report of this association). Patients with Isaacs' syndrome<sup>[9,10]</sup> usually improve after treatment of an underlying cancer or with symptomatic treatment, although evidence is based on case reports<sup>[1]</sup>. Carbamazepine, phenytoin, lamotrigine and sodium valproate can be used, if necessary in combination<sup>[1]</sup>. In patients whose symptoms are debilitating or refractory to symptomatic therapy, immunomodulatory therapies should be tried<sup>[1]</sup>. There are no trials of long-term oral immunosuppression. Prednisolone, with or without azathioprine or methotrexate, has been used in some patients<sup>[1]</sup>. Single case studies suggest that plasma exchange and intravenous immunoglobulins may produce some clinical improvement<sup>[1]</sup>. Of note, all the case studies reporting the effects of the above mentioned immunomodulatory approaches predated the discovery of anti-Caspr2 antibodies<sup>[3]</sup>. Therefore, the immunological serotype of these patients was unknown. Further studies are strongly needed to clarify if the serotype influence the optimal therapeutic approach.

Our report suggests that methylprednisolone 1000 mg/day × 5 days and consecutive tapering followed by plasma exchange can be effective and well tolerated in patient with Isaacs' syndrome due to anti-Caspr2 antibodies. Since the incidence of this condition is rare, controlled clinical studies are not likely to be conducted. Therefore, it is important to report single observations; more cases will be necessary to confirm (or not) the positive effect of this immunomodulatory schedule in anti-Caspr2 Isaacs' syndrome.

## DECLARATIONS

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### Authors' contributions

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Performed the electromyographic examinations: Cafforio G

Performed the plasma exchange procedure: Margelli M

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None.

## Conflicts of interest

There are no conflicts of interest.

## Patient consent

The manuscript does not contain patient identifiable data.

## Ethics approval

Not applicable.

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Original Article

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# Depression severity and its predictors among multiple sclerosis patients in Saudi Arabia: a cross-sectional study

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## Abstract

**Aim:** To assess depression severity among multiple sclerosis (MS) patients.

**Methods:** Our survey was carried out among a sample of 598 MS patients (35.8% were males and 64.2% were females) from all different regions of KSA. A self-administered questionnaire was used for data collection. The Chi-square test was applied to examine the association between demographic factors, depression severity and level of disability.

**Results:** The mean age of patients at the time of diagnosis was 26.1  $\pm$  7.9 years (range 15 to 60 years). The mean duration of the disease was 6.6  $\pm$  4.8 years. More than quarter of patients (27.1%) were admitted during last year. Our results revealed that 9.7% of MS patients had a positive family history of MS, 27.8% of patients were also suffering from different chronic diseases. A large proportion of patients were receiving drugs for MS (e.g. interferon- $\beta$  by 26.2% of patients). Among respondents, the majority (53.2%) were likely to have a mild level of disability and mild depression severity (30.8%), with a significant relationship between the level of disability and depression severity.

**Conclusion:** Severity of depression is mostly mild among MS patients, while only some have severe depression. Depression severity is significantly related to the level of MS patients' disability. Early support of MS patients, especially



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newly diagnosed ones, is strongly advised in order to ensure a better quality of life. It is recommended to conduct a nationwide study to explore severity of depression among MS patients in Saudi Arabia.

**Keywords:** Depression, multiple sclerosis, Saudi Arabia

## INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease, with variable severity and evolution<sup>[1]</sup>. It is a disease that affects the central nervous system, especially the brain, optic nerves and the spinal cord<sup>[2,3]</sup>. Most commonly, MS evolves in relapses<sup>[4]</sup> during symptoms recur or new symptoms occur<sup>[5]</sup>. After a few years, the relapses leave sequelae<sup>[2]</sup> (i.e. permanent symptoms), which can become severe disability. The disease can affect many functions, e.g. movement control, sensory perception, memory, speech, *etc.*<sup>[6]</sup>.

In term of MS epidemiology, there were few studies about the situation in Arabian Gulf countries. Bohlega *et al.*<sup>[7]</sup> reported an increasing incidence of MS, designating the Gulf region as a moderate to high-prevalence zone. The results from studies that focused on MS patients showed an increasing incidence of the pathology<sup>[7-9]</sup>. Prevalence of MS was estimated by Bohlega *et al.*<sup>[7]</sup> to be 40/100,000, who also stated that MS may be under-diagnosed.

Depression has been reported as one of the most common symptoms of MS with a risk of major depressive disorder of 13%-30% and about 50% of lifetime prevalence<sup>[10]</sup>. A direct physical link between depression and multiple sclerosis has been reported. By studying atrophied areas of the brain of MS patients and healthy people by magnetic resonance imaging, researchers could establish a concrete link. It seems that the hippocampus was of lower volume in patients with MS. By analyzing saliva samples, the researchers also noticed that the level of cortisol was particularly high in people with MS. Atrophy of the hippocampus and a high level of cortisol in the body are biological parameters frequently associated with major depressive episodes<sup>[11]</sup>.

The importance of depression among MS patients is unquestionable<sup>[12]</sup>, as this symptom influences the general health and the quality of life of MS patients<sup>[13]</sup>. Therefore, focusing on studying the association between MS and depression seems quite important.

This study aimed to assess the severity of depression and its predictors among multiple sclerosis patients in Saudi Arabia.

## METHODS

This study followed a cross-sectional study which design to test the hypothesis that some patients' sociodemographic (e.g. age, gender, nationality, marital status, monthly income) and other characteristics (e.g. received treatment or grade of disability) may be associated with higher grades of severity of depression among MS patients.

### Study area

During the period from November 2016 to May 2017, this study has been conducted by all geographical regions of Saudi Arabia (i.e. the Southern, Northern, Eastern, Western, and Middle regions).

### Study population and sampling

According to the Saudi Arabian National Multiple Sclerosis Registry<sup>[14]</sup>, there are 2313 MS patients in Saudi Arabia. Following a simple random sample, with a sampling fraction of 1/3, we invited 763 patients (coverage = 33%), only 598 MS patients participated in this study (response rate = 78.4%).

### Data collection tool

This study used a pretested, pre-coded, self-administered questionnaire that included sociodemographic patients' characteristics and Patient Determined Disease Steps (PDDS) to quantify disability in MS patients as well as the Patient Health Questionnaire (PHQ) for quick assessment of depression score among MS patients.

The severity of the illness was measured using the PDDS<sup>[15]</sup>. It is a 9-item patient-administered measure of MS-related disability. Its content validity is indicated by the consistency of the items with the Expanded Disability Status Scale (EDSS)<sup>[16]</sup>. The PDDS scores range from 0 to 8, and can be used to categorize participants into 3 groups according to level of disability: a score of 0 to 2 indicates mild disability, represented by sensory symptoms but no limitations on walking; a score of 3 to 5 indicates moderate disability, represented by symptoms that interfere with daily activities, especially walking, and the need for a cane; and a score of 6 to 8 indicates severe disability, represented by the need for bilateral support, the use of a wheelchair, or being bed-ridden<sup>[17]</sup>.

Learmonth *et al.*<sup>[15]</sup> reported that the PDDS had a strong correlation with the EDSS, supporting criterion aspects of validity. The magnitude and pattern of correlations between PDDS and EDSS scores were consistent between persons with mild and moderate-to-severe disability. Such results provide evidence for the validity of PDDS scores as a patient-reported outcome of disability in persons with MS.

Kroenke *et al.*<sup>[18]</sup> stated that the PHQ is a reliable and valid measure of depression severity. It scores each of the 9 DSM-IV criteria as "0" (not at all) to "3" (nearly every day).

### Data collection method

The questionnaire sheets were personally distributed by researchers to all participant MS patients.

### Data analysis

Collected data were analyzed by using the Statistical Package for Social Sciences (SPSS version 22). We used Chi-square test to examine the association between demographic factors, depression severity and level of disability. *P*-values less than 0.05 were considered statistically significant.

### Ethical considerations

The ethical approval for conducting this study was obtained from Head of Research Ethics Committee (HA-06-B-001) in King Khalid University (REC) # 2016-08-23.

Prior to interviewing participants, the purpose of the study has been explained briefly and their consent has been obtained and they were informed that they have the full right to withdraw at any point of time. Participants' confidentiality and anonymity were fully secured. Finally, participants have been reassured that they have the right to withdraw at any point of time.

## RESULTS

### Demographics of the studied subjects

Participants' socio-demographic characteristics are shown in Table 1. Our study included 598 patients with MS. Males constituted 35.8% of patients. Patients' age ranged between 15 and 60 years with a mean age ( $\pm$  SD) of  $32.4 \pm 8.5$  years. Most participants (87%) were Saudi. About two thirds (63.2%) had a Bachelor Degree, while 24.3% were secondary school educated. More than half of respondents (51.8%) were married and the monthly income of 43.5% was less than 3000 Saudi Riyals (SR).

**Table 1. Demographic characteristics of the participants**

Characteristics		Frequency	Percent (%)
Gender	Male	214	35.8
	Female	384	64.2
Marital status	Single	250	41.8
	Married	310	51.8
	Divorced	36	6.0
	Widower	2	0.3
Nationality	Saudi	520	87.0
	Non-Saudi	78	13.0
Educational level	Illiterate	4	0.7
	Primary	12	2.0
	Intermediate	28	4.7
	Secondary	140	23.4
	University	378	63.2
Monthly income	Postgraduate	36	6.0
	< 3000 SR	260	43.5
	3001-6000 SR	76	12.7
	6001-10,000 SR	130	21.7
	> 10,000 SR	132	22.1
Region	South	170	28.4
	Middle	150	25.1
	East	114	19.1
	North	28	4.7
	West	136	22.7

### Clinical variables

**Table 2** shows the distribution of participants by their clinical variables. The mean duration of the disease ( $\pm$  SD) was  $6.6 \pm 4.8$  years. More than one-fourth of patients (27.1%) were admitted once during last year. Patients were diagnosed with MS at a mean age ( $\pm$  SD) of  $26.1 \pm 7.9$  years. The great majority of respondents (90.3%) had no family history of MS. About the three-quarters of surveyed patients (74.2%) had no associated chronic diseases, while the rest was reported suffering from asthma (4.7%), hypertension (3%), depression (2.7%), and some of them were taking drugs such as interferon beta-1b (Betaferon) (26.2%), followed by interferon beta-1a (Rebif) (20.4%), Fingolimod (Gilenya) (19%). About one-fourth of patients (27.4%) have been diagnosed with depression before and 18.1% were taking anti-depressant drugs.

**Table 3** shows that regarding the PDDS calculated score, more than half of patients (53.2%) were likely to have a mild disability, while 35.5% were likely to have a moderate disability and 11.4% to have a severe disability.

Based on the depression score (PHQ), **Table 4** shows that almost one-third of patients (30.8%) were likely to have mild depression, 24.7% were likely to have moderate depression, 10.7% were likely to have severe depression, while 2.3% appeared to have no depression.

### Relation between patients' sociodemographic factors and depression

Regardless of the type of depression, the prevalence of depression was significantly higher among women than men ( $P < 0.001$ ). Prevalence of depression was significantly higher among patients aged 26-35 years ( $P = 0.016$ ). The severity of depression did not differ significantly according to patients' marital status or nationality. Prevalence of depression differed significantly according to patients' educational level ( $P < 0.001$ ), being higher among those with Bachelor Degree, followed by those with secondary qualification and lower among illiterate patients. Prevalence of depression was significantly higher among patients with the lowest income ( $< 3000$  SR,  $P = 0.001$ ) [**Table 5**].

Severity of depression among participants differed significantly according to their region ( $P < 0.001$ ). Severe depression was highest among patients living in the northern region (28.6%).

**Table 2. Distribution of respondents by clinical variables**

Characteristics	Patients (n = 598)
Duration of the disease (years), mean $\pm$ SD (range)	6.6 $\pm$ 4.8 (0.3-30)
Number of admissions during the last year, n (%)	
0	234 (39.1)
1	162 (27.1)
2	90 (15.1)
3	42 (7)
4	16 (2.7)
5	16 (2.7)
> 5	38 (6.4)
Total	598 (100.0)
Patient age at time of diagnosis (years), mean $\pm$ SD (range)	26.1 $\pm$ 7.9 (0-56)
Number of attacks in the last 2 years, mean $\pm$ SD (range)	1.9 $\pm$ 2 (0-14)
Family history of MS, n (%)	
Yes	58 (9.7)
No	540 (90.3)
Chronic disease, n (%)	
No	442 (74.2)
DM	16 (2.7)
HTN	18 (3)
Asthma	28 (4.7)
Depression	16 (2.7)
Thyroid disease	18 (3)
SLE	2 (0.3)
Anti phospholipid syndrome	16 (2.7)
Behjet	0 (0.0)
Headache/migraine	16 (2.7)
Shogren syndrome	2 (0.3)
More than 1	38 (6.4)
Current drug, n (%)	
Interferon beta-1b (Betaferon)	146 (26.2)
Interferon beta-1a (Rebif)	114 (20.4)
Interferon beta-1a (Avonex)	102 (18.3)
Copaxone (Glatiramer)	0 (0.0)
Teriflunomide (Aubagio)	20 (3.6)
Dimethyl fumarate (Tecfidera)	18 (3.2)
Fingolimod (Gilenya)	106 (19)
Natalizumab (Tysabri)	46 (8.2)
Mitoxantrone	0 (0.0)
Rituximab (Rituxan)	4 (0.7)
Alemtuzumab (Lemetrada)	2 (0.4)
Have been diagnosed with depression before, n (%)	
Yes	164 (27.4)
No	434 (72.6)
Use of anti-depressant drugs, n (%)	
Yes	108 (18.1)
No	490 (81.9)

MS: multiple sclerosis; DM: diabetes mellitus; HTN: hypertension; SLE: systemic lupus erythematosus

**Relation between patients' received medications and depression**

The severity of depression differed significantly according to received medications ( $P < 0.001$ ). All patients who received alemtuzumab had severe depression (2, 100%). Moreover, the highest percentages of moderately severe and severe depression were observed among those who received interferon beta-1a (21.1% and 14%, respectively) and dimethyl fumarate (DMF) (22.2% and 11.1%, respectively) [Table 6].

**Relation between patients' level of disability and depression**

There was a significant association between patients' level of disability and severity of depression ( $P < 0.001$ ). It is to be noted that none of the patients with absent depression had a moderate or severe disability, while those with moderately severe or severe depression had their highest percentages of severe disability (35.3% and 23.5%, respectively) [Table 6].



**Table 3. Distribution of participants by level of disability**

Patient determined disease steps score	Frequency	Percent (%)
Mild (0-2)	318	53.2
Moderate (3-5)	212	35.5
Severe (6-8)	68	11.4
Total	598	100.0

**Table 4. Distribution of participants by depression severity**

Depression score and severity	Frequency	Percent (%)
No (0)	14	2.3
Minimal (1-4)	98	16.4
Mild (5-9)	184	30.8
Moderate (10-14)	148	24.7
Moderately severe (15-19)	90	15.1
Severe (20-27)	64	10.7
Total	598	100.0

## DISCUSSION

Our study illustrated the severity MS disease among the surveyed patients using the PDDS score and the depression severity using the PHQ score. Accordingly, almost half of participants had a PDSS score of 3 or more (i.e. considered to have a moderate or severe disability, 35.5%, and 11.4%, respectively) and more than one-fourth of them were considered to have moderately severe or severe depression.

These findings are in accordance with those of Siegert and Abernethy<sup>[10]</sup>, who reported that depression is one of the most frequently discovered psychiatric symptoms among MS patients. Moreover, Kister *et al.*<sup>[19]</sup> reported that the proportion of MS patients with PDDS score = 3 or more (i.e. moderate to severe disability) reached 50% after 15 years of disease and 75% after 45 years.

Our study showed that about one-third of participants were males. About half of patients were married and the monthly income of almost half of them (43.5%) was less than 3000 SR.

The female predilection observed among participants in the present study has been reported by Kingwell *et al.*<sup>[20]</sup>, who stated that, in the majority of studies, the prevalence of MS was higher in women, with gender ratios ranging from 1.1 to 3. The high prevalence of MS-related disability among our patients may explain why only about half of them are married and almost half of them have low monthly income (i.e. less than 3000 SR).

Results of the present study revealed that prevalence of depression differed significantly with our patients' age and was significantly higher among women than men.

These findings are in accordance with those of Van de Velde *et al.*<sup>[21]</sup>, who noted that depression is significantly associated with gender and age. Women typically have a two-fold higher risk of major depression compared to men. Moreover, Andrade *et al.*<sup>[22]</sup> reported that prevalence of major depression is significantly associated with younger age.

Findings of the present study revealed that severity of depression differed significantly according to patients' educational level, being higher among those with higher education and lower among less educated patients. Moreover, depression was significantly higher among patients with the lowest income (i.e. with monthly income < 3000 SR).

Similarly, Kessler and Bromet<sup>[23]</sup> reported that the poorest respondents in the WHO World Mental Health surveys which were carried out in the USA and several European countries, showed about twofold increased

**Table 5. Severity of depression among multiple sclerosis patients according to their sociodemographic characteristics**

Characteristics	Severity of depression, <i>n</i> (%)						Pvalue
	Absent	Minimal	Mild	Moderate	Moderately severe	Severe	
Gender							< 0.001
Male	8 (3.7)	32 (15.0)	80 (37.4)	62 (29.0)	26 (12.1)	6 (2.8)	
Female	6 (1.6)	66 (17.2)	104 (27.1)	86 (22.4)	64 (16.7)	58 (15.1)	0.016
Age groups							
15-25 years	2 (1.4)	24 (17.4)	42 (30.4)	30 (21.7)	22 (15.9)	18 (13.0)	
26-35 years	8 (3.3)	40 (16.3)	82 (33.3)	62 (25.2)	34 (13.8)	20 (8.1)	
36-45 years	4 (2.3)	24 (13.8)	54 (31.0)	48 (27.6)	30 (17.2)	14 (8.0)	
> 45 years	0 (0.0)	10 (25.0)	6 (15.0)	8 (20.0)	4 (10.0)	12 (30.0)	0.585
Marital status							
Single	6 (2.4)	40 (16.0)	72 (28.8)	68 (27.2)	40 (16.0)	24 (9.6)	
Married	8 (2.9)	54 (17.4)	98 (31.6)	72 (23.2)	42 (13.5)	36 (11.6)	
Divorced	0 (0.0)	4 (11.1)	14 (38.9)	6 (16.7)	8 (22.2)	4 (11.1)	
Widow	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0.340
Nationality							
Saudi	12 (2.3)	84 (16.2)	160 (30.8)	136 (26.2)	76 (14.6)	52 (10.0)	
Non-Saudi	2 (2.6)	14 (17.9)	24 (30.8)	12 (15.4)	14 (17.9)	12 (15.4)	< 0.001
Education							
Illiterate	0 (0.0)	0 (0.0)	2 (50.0)	0 (0.0)	0 (0.0)	2 (50.0)	
Primary	0 (0.0)	6 (50.0)	0 (0.0)	0 (0.0)	4 (33.3)	2 (16.7)	
Intermediate	0 (0.0)	10 (35.7)	2 (7.1)	8 (28.6)	6 (21.4)	2 (7.1)	
Secondary	0 (0.0)	20 (14.3)	46 (32.9)	26 (18.6)	28 (20.0)	20 (14.3)	
University	14 (3.7)	50 (13.2)	122 (32.3)	108 (28.6)	50 (13.2)	34 (9.0)	
Postgraduate	0 (0.0)	12 (33.3)	12 (33.3)	6 (16.7)	2 (5.6)	4 (11.1)	< 0.001
Monthly income							
< 3000 SR	0 (0.0)	32 (12.3)	60 (23.1)	74 (28.5)	52 (20.0)	42 (16.2)	
3001-6000 SR	2 (14.3)	16 (21.1)	22 (28.9)	12 (15.8)	16 (21.1)	8 (10.5)	
6001-10,000 SR	8 (57.1)	20 (15.4)	56 (43.1)	30 (23.1)	8 (6.2)	8 (6.5)	
> 10,000 SR	4 (28.6)	30 (22.7)	46 (34.8)	32 (24.2)	14 (10.6)	6 (4.5)	< 0.001
Region							
Southern	6 (3.5)	28 (16.5)	58 (34.1)	30 (17.6)	26 (15.3)	22 (12.9)	
Middle	4 (2.7)	38 (25.3)	42 (28.0)	38 (25.3)	22 (14.7)	6 (4.0)	
Eastern	4 (3.5)	20 (17.5)	28 (24.6)	28 (24.6)	20 (17.5)	14 (12.3)	
Northern	0 (0.0)	0 (0.0)	10 (35.7)	8 (28.6)	2 (7.1)	8 (28.6)	
Western	0 (0.0)	12 (8.8)	46 (33.8)	44 (32.4)	20 (14.7)	14 (10.3)	

SR: Saudi Riyals

odds of major depressive episodes compared with those in the highest income group. Moreover, in Japan and China (Shenzhen) the least educated had the lowest risk of depression.

The present study showed that severity of depression among MS patients differed significantly according to their region, with the highest percentage of severe depression among those living in the northern region.

These findings necessitate further studies to explore the reason for the significant differences in severity of depression among MS patients according to their location within Saudi Arabia.

Results of the present study showed that severity of depression differed significantly according to received medications. The patients in two cases who received alemtuzumab had severe depression, while the highest percentages of those with moderately severe and severe depression were among those who received interferon beta-1a and DMF.

The significant differences in severity of depression among our MS patients according to the received medication may be attributed to the depression-related side effects associated with those medications.

The current study revealed a significantly positive association between patients' level of disability and the severity of depression.

**Table 6. Severity of depression among multiple sclerosis patients according to their received drugs and disability level**

Variables	Severity of depression, <i>n</i> (%)						<i>P</i> value
	Absent	Minimal	Mild	Moderate	Moderately severe	Severe	
Drugs							< 0.001
Betaferon	8 (5.5)	28 (19.2)	44 (30.1)	32 (21.9)	22 (15.1)	12 (8.2)	
Rebif	2 (1.8)	18 (15.8)	30 (26.3)	24 (21.1)	24 (21.1)	16 (14.0)	
Avonex	0 (0.0)	24 (23.5)	28 (27.5)	20 (19.6)	16 (15.7)	14 (13.7)	
Aubagio	0 (0.0)	0 (0.0)	6 (30.0)	12 (60.0)	2 (10.0)	0 (0.0)	
Tecfidera	0 (0.0)	4 (22.2)	4 (22.2)	4 (22.2)	4 (22.2)	2 (11.1)	
Gilenya	4 (3.8)	10 (9.4)	44 (41.5)	22 (20.8)	14 (13.2)	12 (11.3)	
Tysabri	0 (0.0)	2 (4.3)	14 (30.4)	24 (52.2)	4 (8.7)	2 (4.3)	
Rituxan	0 (0.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)	0 (0.0)	
Lemetrada	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	
Disability level							< 0.001
Mild	14 (4.4)	78 (24.5)	98 (30.8)	76 (23.9)	38 (11.9)	14 (4.4)	
Moderate	0 (0.0)	16 (7.5)	74 (34.9)	60 (28.3)	28 (13.2)	34 (16.0)	
Severe	0 (0.0)	4 (5.9)	12 (17.6)	12 (17.6)	24 (35.3)	16 (23.5)	

The significant association between disability and depression has been emphasized by several studies. Noh *et al.*<sup>[24]</sup> noted that physical disability is significantly related to depressive symptoms. People with physical disability experience multiple risk factors for depressive symptoms, including stereotypic social and personal attitude, abuse, loss of roles, and stressors related to poverty, environmental barriers, and/or lack of access to appropriate health care. Hughes *et al.*<sup>[25]</sup> added that substantial evidence shows that people living with physical disabilities are at least three times more likely to experience depression compared to the general population.

In conclusion, the severity of depression is mostly mild among MS patients, while only some have severe depression. Depression severity is significantly related to the level of MS patients' disability. Early support of MS patients, especially newly diagnosed ones, is strongly advised in order to ensure a better quality of life. It is recommended to conduct a nationwide study to explore severity of depression among MS patients in Saudi Arabia.

## DECLARATIONS

### Authors' contributions

Conception: Alhazzani AA, Ogran H, Abuhawi OH, Al-Hanash AM

Design: Alhazzani AA, Alqahtani MS

Supervision: Alhazzani AA

Materials: Alqahtani MS, AlQahtany RA, Alfaifi AA, Asiri AA, Asiri MA,

Data collection and/or processing: Abuhawi OH, Asiri AY, Al-Hanash AM, AlQahtany RA, Alfaifi AA, Asiri AA

Analysis and/or interpretation: Alqahtani MS, Ogran H, Abuhawi OH, Al-Hanash AM, AlQahtany RA, Asiri AA, Asiri MA

Literature review: Ogran H, Asiri AY, Alfaifi AA, Asiri AA, Asiri MA

Manuscript writing: Alhazzani AA, Asiri AY, AlQahtani MS

Critical review: Alhazzani AA

### Data source and availability

Corresponding author may be contacted for any data inquiries.

### Financial support and sponsorship

None.

## Conflicts of interest

There are no conflicts of interest.

## Patient consent

Consent has been obtained from all participants prior to interviewing. Participants' confidentiality and anonymity were fully secured.

## Ethics approval

The ethical approval for conducting this study was obtained from Head of Research Ethics Committee (HA-06-B-001) in King Khalid University (REC) # 2016-08-23.

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Original Article

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# Applying clustering coefficient to the pattern of international author collaboration in neuroimmunology and neuroinflammation

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## Abstract

**Aim:** To apply cluster coefficient (CC) to the pattern of international author collaborations in neuroimmunology and neuroinflammation using data from Medline and to visualize results using Google maps and social network analysis (SNA).

**Methods:** Selecting 2799 abstracts, author names, countries, and keywords on January 22, 2018 from Medline based on keyword neuroimmunology (or neuroinflammation) within the article title since 1982, we reported following features: (1) nation distribution for the 1st author's nationality; (2) eminent journals and authors in the field of neuroimmunology and neuroinflammation; (3) notable keywords defined by authors representing both neuroimmunology and neuroinflammation; and (4) CCs in networks. We programmed Microsoft Excel VBA routines to extract data from Medline and used Google Maps and SNA Pajek software to display graphical representations with an easy-to-read feature for readers.

**Results:** We found that: (1) the most number of papers in neuroimmunology and neuroinflammation are from the USA (902, 32.23%) and China (363, 12.97%); (2) the productive journals and authors in neuroimmunology and neuroinflammation are *J Neuroinflammation* and *PLoS One*, and Michael T. Heneka (Germany) and Richard M. Ransohoff (USA); (3)



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the most linked keywords are interleukin (IL), IL-1beta, and blood brain barrier; (4) author networks present higher CC than those nation networks.

**Conclusion:** SNA provides wide and deep insight into the relationships among nations in co-author collaboration. The results can help readers in future submission to a journal in neuroimmunology and neuroinflammation.

**Keywords:** Authorship collaboration, Google Maps, neuroimmunology, neuroinflammation, social network analysis

## INTRODUCTION

Neuroimmunology is a field combining neuroscience, the study of the nervous system, and immunology in a review of the immune system. Neuroimmunologists seek to better understand the interactions of these two complex systems during the development of homeostasis and response to injuries. A long-term goal is to further develop our understanding of the pathology of certain neurological diseases<sup>[1]</sup>.

Similarly, neuroinflammation is inflammation of the nervous tissue. It is initiated in response to a variety of cause such as infection, traumatic brain injury, toxic metabolites, or autoimmunity<sup>[2]</sup>. The central nervous system is typically an immunologically privileged site because peripheral immune cells are blocked by the blood-brain barrier<sup>[3,4]</sup>. However, the issues including author collaboration and keyword defined by authors are still unclear.

By January 22, 2018, more than 18,282 papers have appeared on PubMed when searching with the keyword (neuroimmunology OR neuroinflammation) and 2885 in the paper title only including the keyword (neuroimmunology [Title] OR neuroinflammation [Title]) since 1982. The issue of which nations dominate the papers published in neuroimmunology and neuroinflammation intrigues us to investigate which keywords are most frequently seen in those articles in the past decades.

Big data is a concept that has evolved from the modern trend of scientism<sup>[5]</sup>. Many data scientists develop ways to discover new knowledge from the vast quantities of increasingly available information<sup>[5]</sup>. Even an apocryphal story was often told to explain the concept of co-occurrence between beer and diaper sales<sup>[6-8]</sup>, the way of finding both beer and diaper sales had a strong correlation on Friday is unclear in the literature. All possible pairs of observed goods or services are worth studying the association, which is similar to the keywords and authors in journal papers. Fortunately, social network analysis (SNA)<sup>[9-11]</sup> can analyze big data for us to investigate the association of any pairs of goods or services in a network.

Authorship collaboration using SNA is an example regarding co-authors in recent years<sup>[9]</sup> because co-authors among researchers form is a type of social network. Whether the authoring network earns a higher centrality measure (or density) than the national system is required to explore. We are thus interested in using SNA to explore the features in neuroimmunology and neuroinflammation from published papers we observed in Medline library.

Google Maps provide an overall view of geospatial visualization with coordinates of latitude and longitude on a map<sup>[12,13]</sup>. However, a few appeared in Medline library in search of keyword “google map [Title]” on November 22, 2017. Many papers<sup>[9-11]</sup> have studied on co-author collaboration in academics, however, none display results with Google Maps and SNA.

Our aims are to apply the clustering coefficient<sup>[14]</sup> to the pattern of international author collaboration in neuroimmunology and neuroinflammation on the following topics: (1) nation distribution; (2) the most

eminent journals and authors in neuroimmunology and neuroinflammation; (3) the recent research domains defined by authors; and (4) the cluster coefficients (CCs) in different networks.

## METHODS

### Data sources

We programmed Microsoft Excel VBA modules to extract abstracts and their corresponding co-author names as well as author-defined keywords for each article on January 22, 2018, from Medline since 1982. Only those abstracts published by the keyword (neuroimmunology [Title] OR neuroinflammation [Title]) were included. Others like those labeled with Published Erratum, Editorial or without author nation name were excluded from this study. A total of 2885 eligible abstracts were obtained from the Medline.

### Data arrangement to fit SNA requirement

Before visualizing our results using SNA, we organized data in compliance with the format and guidelines defined by Pajek software<sup>[15]</sup>. Microsoft Excel VBA was used to deal with data fitting to the SNA requirement.

### Graphical representations to report

#### *Author nations and their relations*

Two tables (i.e. columns for publication years and rows for the 1st author nations or journals) were made for presenting the distribution in nation (of journal) for the domain of neuroimmunology and neuroinflammation. The bigger bubble means, the more number of the nodes (i.e. nations, or keywords in this study). The wider line indicates, the stronger relations between the 2 nodes. Community clusters are filled with different colors in bubbles. The most eminent authors were calculated from the Medline library since 1982.

#### *Keywords to present the research domain*

If keywords represent the research domain, the stronger relations between the two keywords can be highlighted and linked by SNA. Like the concept of co-occurrence about beer and diaper sales during weekend. The presentation for the bubble and line is similar to the previous section in the interpretation. Keywords defined by the authors were applied to represent the domains in the current study.

### Statistical tools and data analyses

Google Maps<sup>[16]</sup> and SNA Pajek software<sup>[15]</sup> were used to display and visualized representations for eminent authors and keywords in relation with neuroimmunology and neuroinflammation. Author-made Excel VBA modules were applied to organize the data. CC represents the density of a network and a significant level ( $> 1.96$ ) is defined by  $t$ -value as the formula  $[= CC \times [(n-1)/(1-CC^2)]^{1/2}]$ , where  $n$  = the number of nodes in a network.

In contrast, E-I index is defined by the formula, where  $EL$  = the number of external friendship links and  $IL$  = the number of internal friendship links<sup>[17]</sup>. The negative E-I index means a coherence cluster in existence. Similarly, the higher CC indicates many members are members' friends linked to others. Density denotes as the ratio of the linkage members over all possible members.

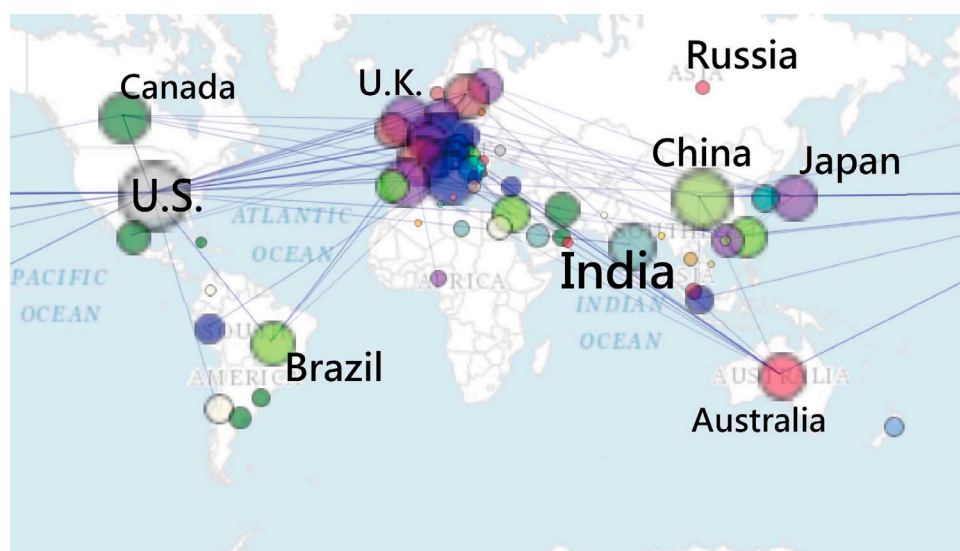
## RESULTS

### Author nations and their relations

A total of 2799 eligible papers with complete author nations based on journal article since 1982 are in [Table 1](#). We can see that the most number of articles are from the countries of USA (902, 32.23%) and China (363, 12.97%). The

**Table 1. Country/region distribution based on the 1st author for papers published in neuroimmunology and neuroinflammation**

Country/region	1982-2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total	%	Growth
USA	148	28	35	41	56	74	81	79	84	115	13	902	32.23	0.42
China	5	2	5	7	12	24	38	47	73	128	17	363	12.97	0.67
Germany	23	4	6	10	9	17	17	20	18	26	1	174	6.22	0.43
Italy	19	2	6	9	11	7	15	9	17	33	4	151	5.39	0.51
Canada	26	3	8	7	7	11	12	13	14	16	1	144	5.14	0.35
India	5		5	4	8	5	9	9	21	24	2	97	3.47	0.57
UK	16	1	2	3	4	3	7	7	11	15	2	87	3.11	0.62
France	12	3	7	1	9	1	4	9	9	15		82	2.93	0.28
Spain	6	2	6	2	4	7	12	7	11	17	1	81	2.89	0.45
The Netherlands	11	2	7	4	8	3	8	3	6	7		70	2.50	-0.12
Brazil	1	1	3	2	1	4	5	12	8	17	6	61	2.18	0.73
Australia	1	1		8	3	5	7	12	6	15	1	60	2.14	0.46
Japan	4	3	1	3	9	2	7	9	7	9	1	59	2.11	0.34
Taiwan	2	3	4	2	8	3	3	6	12	9	1	55	1.96	0.32
Sweden	7	1	5		3	4	4	6	4	7		48	1.71	0.26
Israel	9	2	1	2	2	2	3	2	3	1	1	37	1.32	-0.07
Belgium	3			2	2	2	5	3	1	6	1	28	1.00	0.48
Switzerland	3		1	2	1	3	1	2	5	5	1	27	0.96	0.56
Iran	0			4	2	1	1	5	2	9		24	0.86	0.40
Mexico	2		1		2		2		4	7	1	21	0.75	0.54
Others	17	7	7	8	13	18	23	26	44	45	3	228	8.15	0.55
Total	320	65	110	121	174	196	264	286	360	526	57	2799	100.00	0.55

**Figure 1.** Google Maps on the topic of author collaboration in neuroimmunology and neuroinflammation (cluster coefficient = 0.61)

trend in the number of publications with authorship is present in the column of growth in Table 1. All nations but the Netherlands and Israel showed a positively increase.

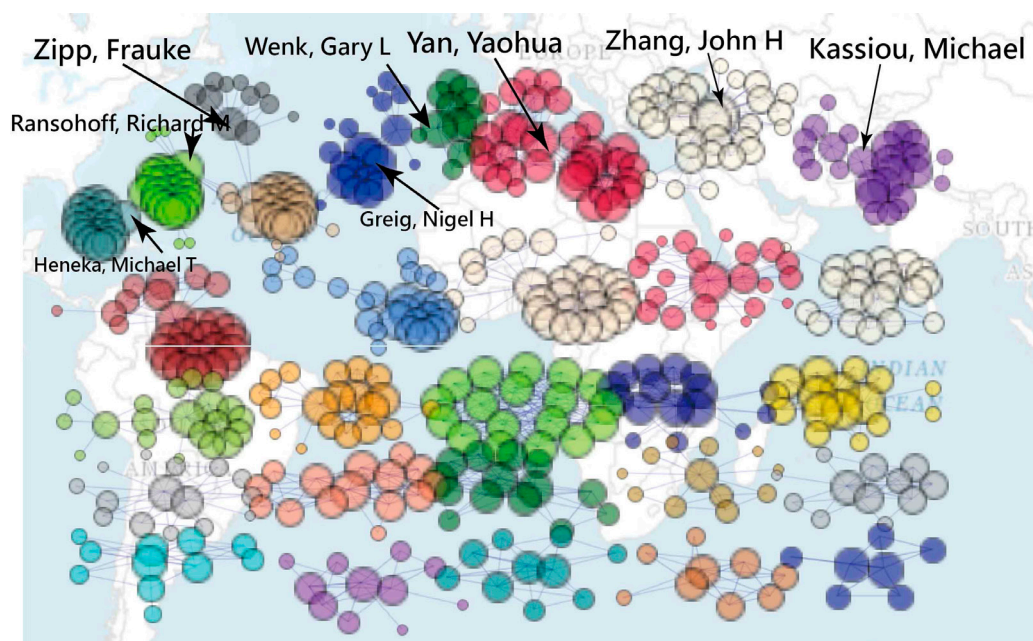
The diagram in Figure 1 displays author collaboration among nations. Overall, the highest production in countries are from the USA, China, and Europe [Figure 1]. Any collaborated with others are shown with a blue line. Interested authors are recommended to click the bubble of interest to see details on a website at the reference<sup>[18]</sup>.

### The eminent authors in surgery

The most prominent journals and authors with the most number of papers in neuroimmunology and neuroinflammation are *J Neuroinflammation* and *PLoS One* as well as Michael T. Heneka (Germany) and

**Table 2. Journal distribution based for papers published in neuroimmunology and neuroinflammation**

Journal	1982-2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total	%	Growth
<i>J Neuroinflammation</i>	12	3	7	7	32	14	28	27	38	34	1	215	7.90	0.42
<i>PLoS One</i>	0	2	2	4	10	20	15	7	5	7	1	288	10.58	0.05
<i>Brain Behav Immun</i>	3	1	1	4	3	2	6	7	12	26	4	145	5.33	0.62
<i>J Neuroimmunol</i>	24	4	1		3	4	6	6	8	8	1	161	5.91	0.46
<i>J Neurosci</i>	10	2	4	2	4	3	4	4	4	4		140	5.14	-0.09
<i>Neuroscience</i>	5	1		3	2	3	4	6	3	9	1	93	3.42	0.52
<i>J Neurochem</i>	10			4	1	1	4	4	5	4	1	86	3.16	0.51
<i>Mol Neurobiol</i>	0	1			2	2	3	2	9	15		78	2.87	0.54
<i>Exp Neurol</i>	5		4	1	1	2	6	3	6	3	1	71	2.61	0.32
<i>Brain Res</i>	5		2	5		2	1	2	6	6	1	72	2.65	0.37
<i>Mediators Inflamm</i>	1					10	4	5	4	6		66	2.42	0.37
<i>Neurochem Res</i>	1			1	3	1	2	4	6	9	2	61	2.24	0.71
<i>Sci Rep</i>	0			1		1		7	9	10		58	2.13	0.58
<i>Neurobiol Dis</i>	7	1	1	1	2	5		1	3	1	3	60	2.20	0.25
<i>J Alzheimers Dis</i>	3	1	5	1	3		2	1	3	6		60	2.20	0.04
<i>J Neuroimmune Pharmacol</i>	2	2	1		3	8	2	1	2	4		55	2.02	0.01
<i>Glia</i>	5		3	2		3	2		4	4		55	2.02	0.17
<i>J Immunol</i>	6	1	1	3		2	5	3		1		56	2.06	-0.13
<i>Neurochem Int</i>	2		2	1	3	3	2	2	3	3		51	1.87	0.20
<i>Front Cell Neurosci</i>	0					1	7	5		6		42	1.54	0.40
Others	250	47	85	92	121	127	206	218	263	394	45	6061	222.67	0.56
Total	351	66	119	132	193	214	309	315	393	560	61	2722	100.00	0.55

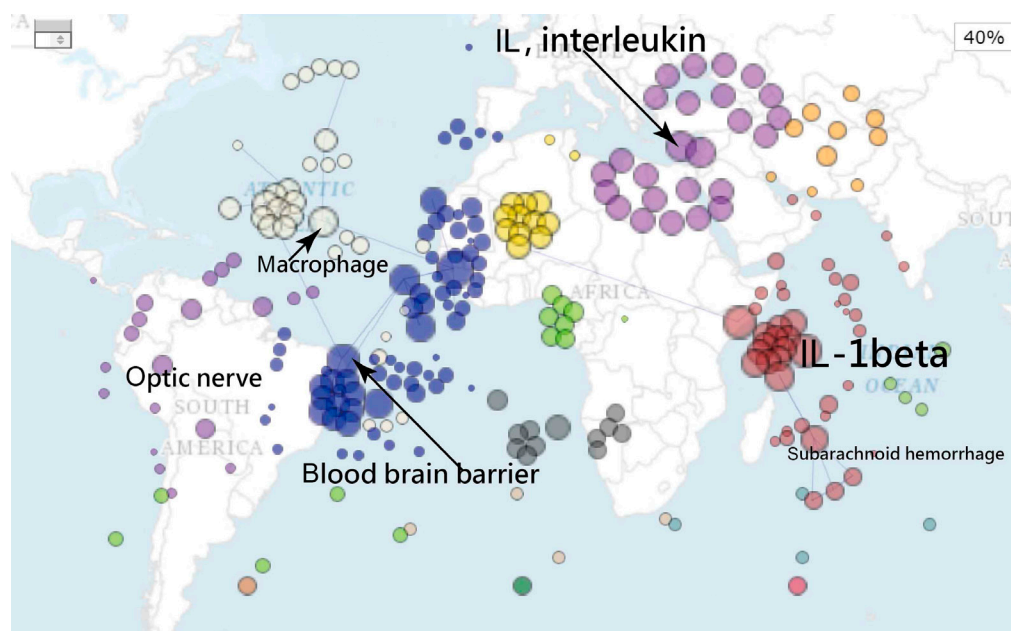
**Figure 2.** Google Maps on eminent authors in neuroimmunology and neuroinflammation (cluster coefficient = 1.0)

Richard M. Ransohoff (USA) [Table 2 and Figure 2]. The link on website appears at <http://www.healthup.org.tw/gps/Neuroimmunologyauthor.htm><sup>[19]</sup>.

### Keywords to present the research domain

The most linked keywords are interleukin (IL), IL-1beta, and blood-brain barrier [Figure 3]<sup>[20]</sup>. We can see that the keywords consisting of many clusters with different cluster coefficients.





**Figure 3.** Google Maps on author-defined keywords in neuroimmunology and neuroinflammation (cluster coefficient = 0.60)

**Table 3.** CCs in each cluster and their relevant indicators

Name	CC	Density	Weighted	EI	Node	Degree	Weighted	t-value
Country/region								
Canada	0.61	0.60	0.80	0.12	6	9	12	1.54
Germany	0.60	0.67	2.00	0.64	4	4	12	1.06
UK	0.57	0.76	2.05	0.20	7	16	43	1.55
Italy	0.21	0.47	0.80	0.36	6	7	12	0.43
France	0.20	0.67	1.83	0.67	4	4	11	0.29
India	0.20	0.67	0.83	0.00	4	4	5	0.29
Australia	0.00	0.5	0.50	0.52	4	3	3	0.00
Brazil	0.00	0.67	1.33	0.65	3	2	4	0.00
Chile	0.00	0.67	0.67	0.20	3	2	2	0.00
USA				1.00	1			
China		1.00	2.00	0.75	2	1	2	
The Netherlands		1.00	6.00	0.85	2	1	6	
Taiwan		1.00	2.00	0.60	2	1	2	
Author								
Michael T. Heneka	1.00	1.00	2.00	-0.99	17	136	272	
Richard M. Ransohoff	0.59	0.62	1.25	-0.97	23	158	316	3.35*
Janine Doorduyn	0.56	0.30	0.72	-1.00	26	97	235	3.31*
Gary L. Wenk	0.52	0.58	1.65	-0.97	12	38	109	1.93
Dong-Kug Choi	0.45	0.56	1.35	-1.00	16	67	162	1.89
Borja Garcia-Bueno	0.43	0.31	0.70	-1.00	20	58	133	2.02*
John A. Olschowka	0.43	0.61	1.97	-1.00	9	22	71	1.26
Tomas Olsson	0.42	0.37	0.88	-1.00	19	64	150	1.91
Vicente Felipe	0.41	0.54	1.94	-1.00	16	65	233	1.68
Stefan Bittner	0.41	0.51	1.06	-1.00	27	179	373	2.25*
Frederic Dolle	0.41	0.26	0.66	-1.00	27	92	231	2.25*

\*Denotes significance when *t*-value greater than 2.0. CC: cluster coefficient.

### Cluster coefficients in a network

Each cluster has its cluster coefficient representing the density of a network. We found that author clusters earn higher CC than have nation clusters. Cluster coefficient has a significant effect in comparison with a considerable *t*-value (> 2.0), indicating author network with more significance than those of the nations [Table 3].

## DISCUSSION

### What this adds to what was known

Many previous types of research<sup>[9-11]</sup> have investigated co-author collaboration using SNA. The results (the most number of articles from the USA and Europe) are similar to the findings that dominant nations in science come from the USA and Europe<sup>[21-23]</sup>, but China recently has an increasing trend in publication. Referring to the apocryphal story told to discover the co-occurrence about beer and diaper sales<sup>[6-8]</sup>, we presented a novel method incorporating SNA with Google Maps to explore the data. It can be seen that visual representations offered to the readers are rare in literature. Traditionally, it is hard to observe the association of two or more symptoms or entities together that appeared in a network at that moment.

Journal authorship collaboration compared with each other using Google Maps is illustrated in this study. We can see that many links are connecting two nations which indicate a collaboration pattern in paper publication similar to the previous study<sup>[7]</sup>. Hence, it is easy to observe the phenomena of international author collaboration in neuroimmunology and neuroinflammation, which is inconsistent with the earlier reviews that investigated scientific collaboration of Iranian Psychology and Psychiatry Researchers<sup>[23,24]</sup>.

There are 319 papers with the keyword of SNA when searching Medline on December 21, 2017. There were only two papers<sup>[25,26]</sup> that incorporated medical subject heading into SNA to release relevant knowledge to readers. However, only a few<sup>[27,28]</sup> include Google Maps link like we used in our current study. The CCs we illustrated at references<sup>[17-20]</sup> are called overall CCs. The CCs in figures are 1.0, 0.61, and 0.60, respectively, which is different from the global CCs or individual CC defined by each cluster or by each node. Evidence suggests that in most real-world networks, nodes tend to create a tightly knit groups characterized by a relatively high density of ties; this likelihood tends to be higher than the average probability of a link randomly established between two nodes<sup>[15,29]</sup>.

### What it implies and what should be changed?

Scientific publication is one of the objective measurements to evaluate the achievements of a medical specialty or discipline<sup>[30]</sup>. It is worth using SNA and Google Maps to explore knowledge to readers in the future.

Many algorithms and measures (or indicators) have been developed in SNA to the graphical exploration of our data. If we investigate any author or paper most fits the research domain in a target journal, the centrality measures can be used<sup>[9]</sup>. It means that the core subject can be analyzed using the centrality measure<sup>[11,24]</sup> yielded by SNA.

### Strengths of this study

The way we incorporated SNA with Google Maps is unique when compared with these published papers<sup>[9-11]</sup> merely using a single method of SNA. Another strength and feature of this study is that with Google Maps linked to the references<sup>[18-20]</sup>, for interested readers, one can manipulate the link in their own ways on the Google dashboards. The national distribution in [Figure 1](#) allow the reader to easily understand the feature of international author collaborations in neuroimmunology and neuroinflammation. One picture is worth ten thousand words. We hope following studies can report other kinds of information using Google API in the future.

### Limitations and future study

The interpretation and generalization of the conclusions of this research should be carried out with caution. First, the data from this study are from Medline for a single journal. It is worth noting that any attempt to generalize the findings of this study should be cautious in the fields of journal domains.

Secondly, although the data were extracted from Medline and carefully dealt with during every linkage as correctly as possible, the original downloaded text file might have some errors in symbols such as period and comma in author address that may lead to some bias in the resulting nation distribution.

Thirdly, there are many algorithms used for SNA. We merely applied separation components showing in figures. Any changes made along the algorithm will cause different pattern.

Fourth, the social network analysis is not subject to the Pajec software we used in this study. Others such as Ucinet<sup>[31]</sup> and Gephi<sup>[32]</sup> are suggested to readers for use in the future.

In conclusion, social network analysis provides wide and deep insight into the relationships among nations in coauthor collaboration. The results can help readers in future submission to a journal in neuroimmunology and neuroinflammation.

## DECLARATIONS

### Authors' contributions

Drafted the manuscript: Hsu CF

Developed the study concept and design: Chien TW

Analyzed and interpreted the data: Chien TW, Hsu CF, Chow JC

Monitored/supervised the process of this study and helped in responding to the reviewers' advice and comments: Chou W

Read and approved the final manuscript: all authors

### Data source and availability

All data were downloaded from Medicine library and in Tables.

### Financial support and sponsorship

None.

### Conflicts of interest

The authors declare that they have no competing interests.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

### Copyright

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Review

Open Access



# MicroRNA-126 is a prospective target for vascular disease

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## Abstract

MicroRNA-126 was involved in angiogenesis during physiological and pathological process. It was mainly expressed in endothelial cells, and defined as a pivotal biological molecule associated with vascular disease. Increased microRNA-126 in endothelial cells promotes angiogenesis in ischemic stroke, repairs impaired endothelial cells in atherosclerosis, and attenuates vascular dysfunction in diabetics. By contrast, microRNA-126 transferred from endothelial cell to smooth muscle cells could lead to proliferation that induced intimal hyperplasia. Additionally, microRNA-126 could be a tumor suppressor or an oncogene, which was depended on the cancer type. In this review, we summarized the function of microRNA-126 in ischemic stroke, atherosclerosis, diabetics, tumor, and discussed the underlying mechanisms.

**Keywords:** Atherosclerosis, diabetes, ischemia, microRNA-126

## INTRODUCTION

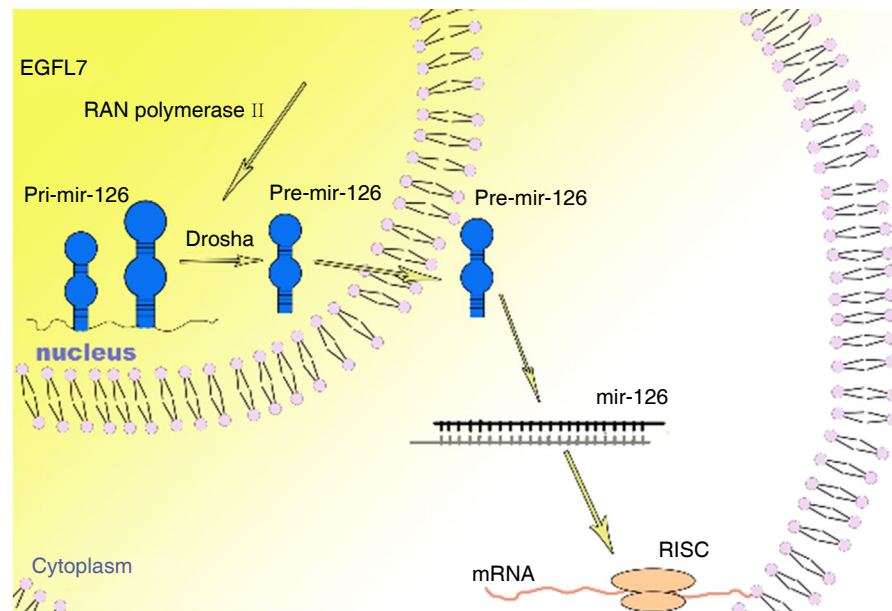
MicroRNAs (miRNAs) are 20-22 nucleotides single-strand and non-coding RNA, which are involved in regulating diverse cellular processes. MiRNAs are originally transcribed from portion of introns of mRNA or independent miRNA genes<sup>[1]</sup>. MiRNAs regulate gene expression through Ago2-RISC or Ago1-RISC, which depends on if miRNA precisely matched with its target sequence<sup>[2]</sup>.

MicroRNA-126 (miR-126) is located in an intron of the epidermal growth factor-like-domain 7 gene (EGFL7). The pri-miR-126 is transcribed from EGFL7 by RNA polymerase II. With the aid of RNase III



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**Figure 1.** MiR-126 is located in the 7th intron of EGFL7. RNA polymerase II binds with EGFL7, resulting in the pri-miR-126 formation. Pri-miRNA-126 is targeted to Drosha for cleavage, giving birth to pre-miR-126. Pre-miR-126 is transferred from the nucleus to the cytoplasm by exporting 5', in which it matures into miR-126. The Dicer cleaves miR-126 into single-strand and either strand of the duplex may potentially act as a functional miRNA, integrating into RISC. The RISC with a microRNA recognizes complementary mRNA molecules and degrades or silences them, resulting in substantially decreased levels of protein translation and effectively inhibiting the gene. EGFL7: epidermal growth factor-like-domain 7 gene; RISC: RNA-induced silencing complex

enzyme Drosha, the pri-miR-126 generates shorter stem-loop precursors (pre-miRNA). Pre-miR-126 is exported to the cytoplasm by exportin-5<sup>[3]</sup>. In the cytoplasm, RNase III enzyme Dicer processes pre-miR-126 into mature 20-24 nucleotide miRNAs, which then incorporate into the RNA-induced silencing complex (RISC, Figure 1).

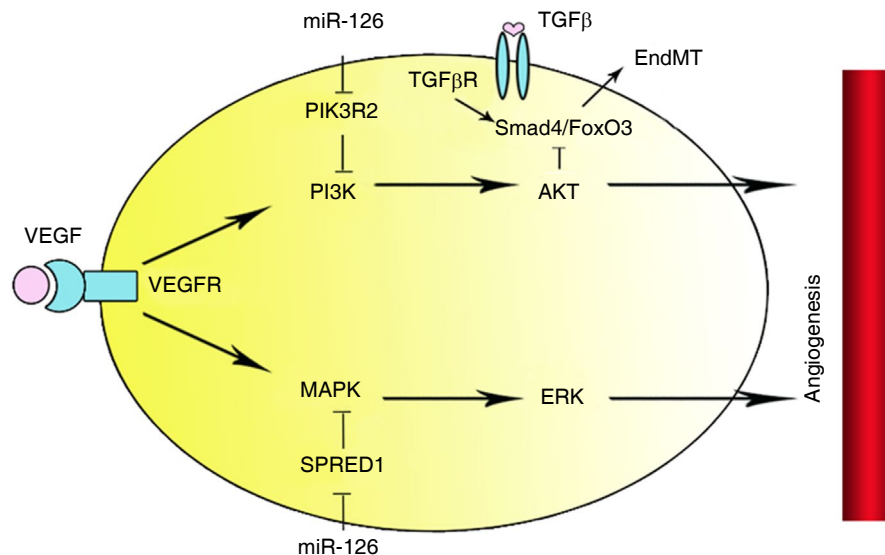
MiR-126 is specifically and highly expressed in endothelial cells (ECs), which regulates ECs migration, cytoskeleton reorganization, capillary network stability, cell survival and apoptosis<sup>[4]</sup>. And microRNA-126 regulates cell survival or apoptosis, depending on different cell types. Furthermore, miR-126 is necessary for the maintenance of vascular structure *in vivo*<sup>[5]</sup>.

## MIR-126 PROMOTES ANGIOGENESIS AFTER ISCHEMIC STROKE

Ischemic stroke was one of the major causes of death and disability worldwide<sup>[6]</sup>. It was classified into large-artery, cardioembolic, and small-vessel (lacunar stroke) ischemic stroke<sup>[7]</sup>. Currently, angiogenesis is regarded as a promising therapy for the repairing and remodeling after ischemic stroke. Angiogenesis is the growth and remodeling process of the primitive vascular network. Angiogenesis is involved in enlargement of pre-existing vessels or formation of capillaries through trans-endothelial cell bridges<sup>[8]</sup>. Angiogenesis attenuated functional deficits and promoted behavioral recovery may through restoring the blood flow in the ischemic area. In recent years, studies demonstrated that deletion of miR-126 in mice embryo and zebra fish embryo reduced the integrity of vessels and decreased postnatal angiogenesis<sup>[5,9]</sup>.

MiR-126 regulated the response of ECs to vascular endothelial growth factor (VEGF), which was via directly repressing negative regulators of VEGF pathway. The negative regulators were the Sprout-related EVH1 domain-containing protein 1 (SPRED1) and phosphoinositide-3 kinase regulatory subunit 2 (PIK3R2)<sup>[5]</sup>. MiR-126 was an important factor, which could maintain the vascular integrity. Antago-miR-126 decreases ischemia-induced angiogenesis in hind-limb ischemia by increasing SPRED1 and PIK3R2 [Figure 2]<sup>[10]</sup>.





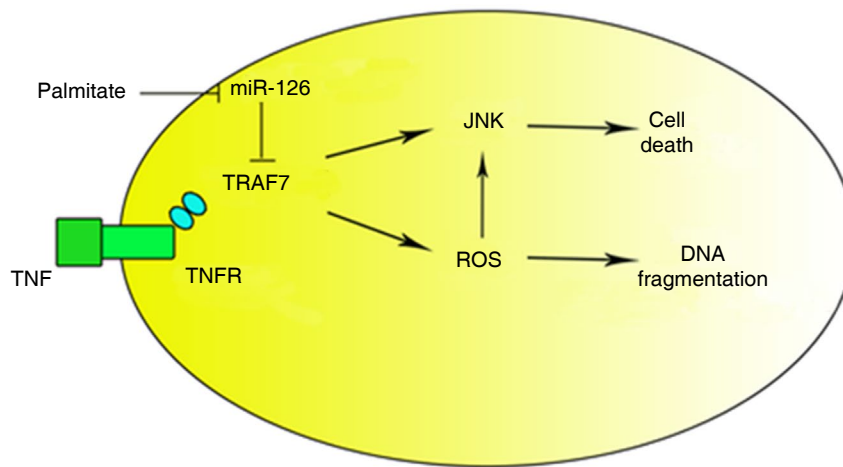
**Figure 2.** In endothelial cells, miR-126 inhibited PIK3R2 and SPRED1 to facilitate angiogenesis by activating PI3K/AKT and MAPK/ERK signal pathway indirectly. In EPCs, miR-126 inhibited EPCs EndMT inducing by TGFb through activating PI3K/AKT signal pathway. EPC: endothelial progenitor cell; VEGF: vascular endothelial growth factor; TGFb: transforming growth factor beta; EndMT: endothelial-to-mesenchymal transition; PIK3R2: phosphoinositide-3 kinase regulatory subunit 2; SPRED1: sprout-related EVH1 domain-containing protein 1

MiR-126 affected the expression of stromal cell derived factor-1 (SDF-1) from different approaches. In normal ECs, miR-126 repressed the SDF-1 synthesis by directly binding to SDF-1 mRNA. Normal miR-126 level was enough to modulate SDF-1 and vascular cell adhesion molecule 1 (VCAM-1) expression in ECs<sup>[11]</sup>. Under high glucose condition associated with ECs dysfunction, decreasing miR-126 could increase SDF-1 expression, and also directly increased progenitor cells migration and adhesion<sup>[11,12]</sup>, and further improve stroke outcome by differentiating into endothelial cells or through the paracrine effects. Tenreiro *et al.*<sup>[13]</sup> demonstrated endothelial cells improved ischemic recovery by differentiation into ECs. Chen *et al.*<sup>[14]</sup> demonstrated progenitor cells secreted IL-8 to promote angiogenesis during ischemia. In contrast, under atherosclerosis miR-126 could elevate C-X-C chemokine receptor type 4 (CXCR4) expression by repressing the function of G protein-coupled receptor (GPCR) signaling inhibitor. As a result, SDF-1 could be up-regulated and recruited progenitor cells to the damaged area<sup>[15]</sup>. In the kidney ischemic condition, miR-126 overexpression in the hematopoietic compartment could attenuate CXCR4 expression on the bone marrow stem cells and at the same time increase SDF-1 in the ischemic tissue. Thus increased SDF-1 facilitated stem cell mobilization towards the ischemic area<sup>[16]</sup>. Taken together, miR-126 plays a protective role during ischemic injury and is a potential target for ischemic stroke therapy.

## MIR-126 PLAYS A DUAL-ROLE IN ATHEROSCLEROSIS

Atherosclerosis was a pathophysiologic process initiated by death of ECs<sup>[17]</sup>. MiR-126 played a vital role in atherosclerosis<sup>[18]</sup>. Studies implicated the regulation of ECs proliferation and repair may reduce atherosclerosis formation<sup>[19]</sup>. In atherosclerosis formation, transferring miR-126 from apoptotic bodies to recipient cells elevated SDF-1, which promoted progenitor cell mobilization and incorporation during plaque formation. Consequently, atherosclerotic progression was impeded<sup>[15]</sup>.

Besides recruiting progenitor cells, miR-126 directly affected ECs proliferation to reduce atherosclerosis<sup>[20]</sup>. MiR-126-5p promoted ECs proliferation and limits atherosclerosis by suppressing the Noth1 inhibitor delta-like 1 homolog (Dlk1)<sup>[20]</sup>. MiR-126 down-regulated VCAM-1 expression, thus decreased leukocyte adhesion and resisted vascular wall inflammation<sup>[21]</sup>. Likewise, up-regulating miR-126 in human aortic ECs



**Figure 3.** TRAF7 activated ROS and JNK pathway. MiR-126 increased ROS and inhibited JNK pathway via binding TRAF7. Palmitate promoted cell death and DNA damaged by inhibiting miR-126. TRAF7: tumor necrosis factor receptor-associated factor 7; TNFR: tumor necrosis factor receptor; ROS: reactive oxygen species

(HAEC) showed a decrease of monocyte adhesion via decreasing VCAM-1 expression<sup>[22]</sup>. In this manner, miR-126 played a beneficial role in atherosclerosis.

MiR-126 targeted numerous putative mRNAs to exert anti-atherosclerosis effect in varied patterns<sup>[18]</sup>. For example, it degraded tumor necrosis factor receptor-associated factor 7 (TRAF7) to protect ECs from injury. TRAF7 bound to the tumor necrosis factor receptor (TNFR) and generate intracellular reactive oxygen species (ROS), or reduce the anti-apoptotic molecule expression. MiR-126 was inhibited by palmitate, a major saturated free fatty acid in plasma<sup>[23]</sup>. MiR-126 overexpression decreased TRAF7 and rescued ECs from saturated free fatty acids (FFAs) damage<sup>[23]</sup>. These studies demonstrated that miR-126 protected ECs from palmitate and relieved oxidative stress, further induced the effect of anti-atherosclerosis [Figure 3]<sup>[24]</sup>.

In general, endothelial progenitor cells (EPCs) differentiated into ECs to repair damaged intima. However, bone marrow derived EPCs also trans-differentiated into a smooth muscle cell lineage, regarded as endothelial-to-mesenchymal transition (EndMT). Smad3 and Smad4 formed a complex with FoxO3, which played an essential role in cell differentiation. MiR-126 activated PI3K/AKT pathway, and indirectly inhibited FoxO3/Smad4, which was associated with EPC EndMT [Figure 2]<sup>[25]</sup>.

Although numerous researches indicated that miR-126 benefited anti-atherosclerosis, studies also demonstrated the adverse effect of miR-126 on atherosclerosis. It was noted that smooth muscle cells (SMCs) could be activated and proliferate in atherosclerosis<sup>[26]</sup>, meanwhile SMC apoptosis caused vulnerable plaques<sup>[27]</sup>. Transmission of miR-126 to SMCs mediated Forkhead box O3 (FoxO3), B-cell lymphoma-2 (BCL2) and insulin receptor substrate-1 (IRS1) expression, thus affected SMC proliferation, cell cycle progression, and apoptosis<sup>[28]</sup>. Zhou *et al.*<sup>[28]</sup> did not detect microRNA-126 in SMC during atherosclerosis, and a recent study showed that microRNA-126 was up-regulated in atherosclerosis mice<sup>[29]</sup>.

Taken together, miR-126 in SMCs exacerbated atherosclerosis despite it had beneficial effects on endothelial functions. Gene regulation of biological processes extremely complex. Therefore, to explore the modulation mechanism between miRNAs and mRNA seems important.

### MIR-126 IS A POTENTIAL TARGET IN DIABETIC ECS DYSFUNCTION

Malfunction of ECs was one of the distinct changes in the arterial wall by diabetes induced metabolic abnormalities<sup>[30]</sup>. Sprout-related EVH1 domain-containing protein 1 (SPRED1) was a Ras/ERK signaling

inhibitor, which was involved in the regulation of several cellular processes such as differentiation, survival, motility and cell cycle<sup>[31]</sup>. MiR-126 affected EPC function via its target SPRED1<sup>[31]</sup>. PI3K/AKT/eNOS pathway played a role in preventing high glucose-induced cell injury<sup>[31]</sup>. As mentioned above, miR-126 activated PI3K/AKT/eNOS signal pathway and rescued EPC function by degrading PIK3R<sup>[5]</sup>. MiR-126 expression was down-regulated in type II diabetic derived EPCs. MiR-126 overexpression in EPCs promoted EPC proliferation, migration, and inhibited EPC apoptosis<sup>[31]</sup>. Experiments demonstrated that endothelial micro-particles derived from glucose-treated ECs had lower amounts of miR-126, which reduced endothelial repair capacity *in vitro* and *in vivo*<sup>[32]</sup>. It could speculate that miR-126 up-regulation in glucose-damaged ECs protected them from glucose-induced dysfunction.

In consistent with above reports, Zampetaki *et al.*<sup>[33]</sup> demonstrated that loss of miR-126 was associated with diabetics. MiR-126 level in endothelial apoptotic bodies was reduced in a glucose-dependent fashion. Low plasma miR-126 level caused VEGF resistance and endothelial dysfunction, which related to diabetics complications<sup>[33]</sup>.

MiR-126 activated VEGF signaling by repressing SPRED1 and PI3R2. Circulating miR-126 has been proposed as a marker for endothelial dysfunction in diabetics. Therefore, up-regulating miR-126 in plasma provided a unique approach for the therapy of endothelial injury.

## MIR-126 AND TUMOR

There was no doubt that miR-126 was related to tumorigenic process. Studies showed that MiR-126 was not only a tumor suppressor, but also an oncogene depending on the type of cancer<sup>[34-39]</sup>. MiR-126 negatively cancer cell proliferation, migration, invasion and survival while it also accelerated cancer progression through the promotion of microvessel formation<sup>[34]</sup>.

Increase of miR-126 was beneficial for the acute myelocytic leukemia (AML) through degrading HOXA9. HOXA9 was an oncogene and often elevated in myelocytic leukemia<sup>[35]</sup>. However, miR-126 overexpression in AML was associated with poor survival and higher chance of relapse<sup>[36]</sup>. Decrease of miR-126 expression in AML cells reduced cell growth by inducing apoptosis *in vitro*<sup>[36]</sup>.

Transferring miR-126 mimics into colon cancer cells reduced cell viability, migration, and invasion by degrading CXCR4<sup>[40]</sup>. SDF-1 binding to CXCR4 activated NF-κB pathway and increased MMP-2, MMP-9, VEGF and nitric oxide expression. These factors promoted tumor cell invasion through degradation of the extracellular matrix and promotion of angiogenesis, hematopoiesis, ECs growth<sup>[41]</sup>.

MiR-126 is an inhibiting microRNA on the tumor development<sup>[38,39]</sup>. Studies demonstrated that VEGF-A was a target of MiR-126, which could down-regulate miR-126 and increase VEGF-A expression in tumors<sup>[38]</sup>. In malignant mesothelioma, miR-126 indirectly increased FOXO1 by targeting IRS1 leading to apoptosis, cell cycle arrest, and stress resistance in various tissues<sup>[39]</sup>.

According to current researches, miR-126 was down-regulated in most tumors such as colorectal cancer, gastric cancer, lung cancer, breast cancer. However, miR-126 was up-regulated in the acute myeloid leukemia<sup>[36]</sup>. MiR-126 could be a tumor marker in a non-invasive diagnostic method<sup>[42]</sup>.

In conclusion, miR-126 is a double-edged sword and plays distinct roles in different cell types and microenvironment. It could be a potential therapeutic target and prognostic biomarker for the vascular disease, diabetics and tumor. Exploring the effects and underlying mechanisms of miR-126 is important and timely.

## DECLARATIONS

### Authors' contributions

Summarized the references and wrote the manuscript: Qu MJ

Reviewed literature and discussed paper writing: Pan JJ, Shi XJ

Discussed paper writing and revised the manuscript: Zhang ZJ, Tang YH

Wrote the outline of the paper, edited and approved the final version of the manuscript: Yang GY

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### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Original Article

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# Acetaminophen metabolites p-aminophenol and AM404 inhibit microglial activation

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## Abstract

**Aim:** Alzheimer's disease (AD) is a neurodegenerative disorder characterized by cognitive decline, deposits of amyloid beta and neurofibrillary tangles. Inflammation facilitated by microglia, the resident immune cells of the brain, contribute to the pathogenesis of AD. Epidemiological data indicate that nonsteroidal anti-inflammatory drugs (NSAIDs), which are cyclooxygenase (COX) inhibitors, reduce the risk of developing AD when administered over the course of two or more years. The mechanisms underlying this protective effect are unknown. Acetaminophen (paracetamol), which is not effective as an inhibitor of COX in peripheral tissues, may provide similar protection without the adverse effects of chronic NSAID use. The beneficial effects of acetaminophen have been proposed to stem from its metabolites p-aminophenol and N-arachidonoylaminophenol (AM404), of which, AM404 possesses analgesic and antipyretic properties. The goal of this study was to compare the effects of acetaminophen and its metabolites on microglial immune function and to elucidate the molecular mechanisms engaged by these compounds.

**Methods:** Lipopolysaccharide-stimulated BV-2 murine microglia were used as models. Microglial activation was monitored by their secretion of nitric oxide.

**Results:** P-aminophenol and AM404 suppressed nitric oxide secretion from stimulated microglia more effectively than acetaminophen through pathways that were independent of COX inhibition, cannabinoid receptor type two (CB2) inhibition, and activation of transient receptor potential cation channel subfamily V member 1 (TRPV1).

**Conclusion:** Since AM404 has been previously demonstrated to attenuate NF- $\kappa$ B activation, it is likely that the protective effects of acetaminophen against adverse microglia activation are mediated by its metabolites p-aminophenol and AM404 inhibiting this transcription factor.



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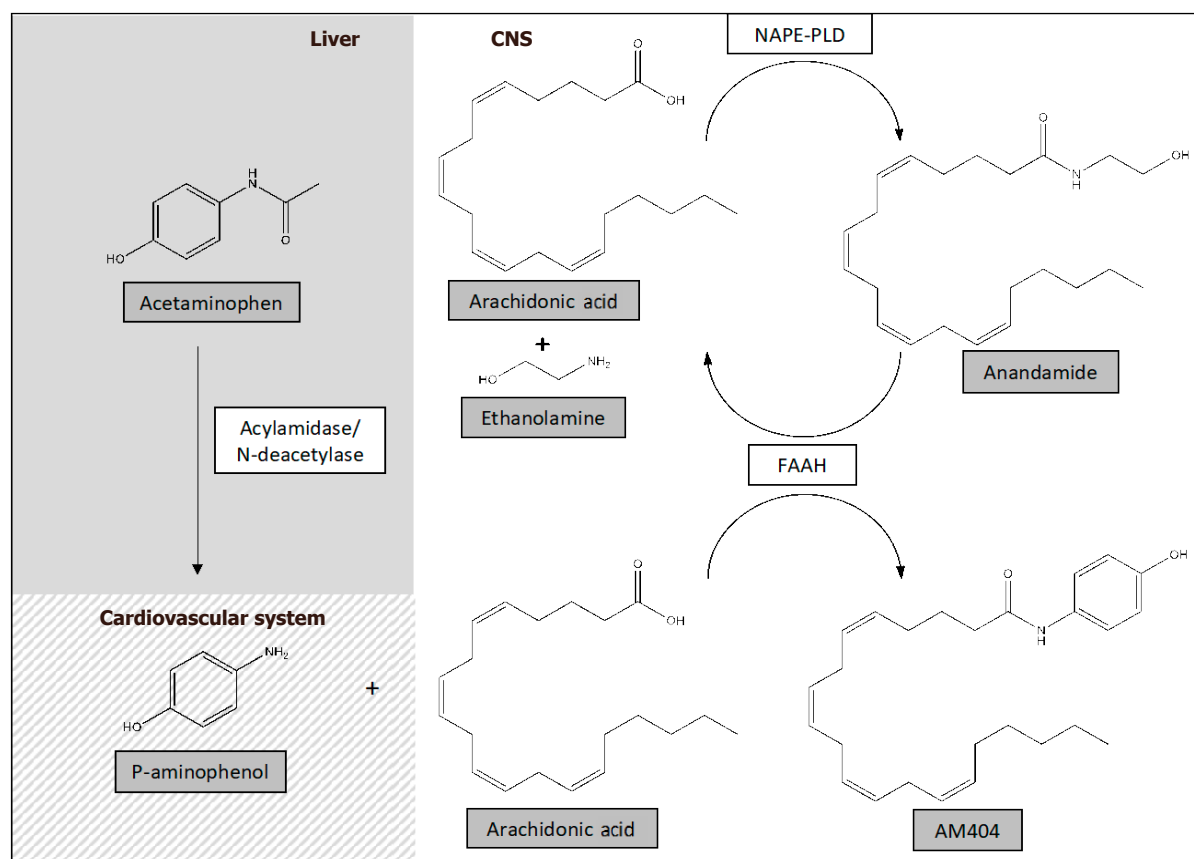
**Keywords:** Acetaminophen, p-aminophenol, AM404, neuroinflammation, pharmacodynamics, microglia

## INTRODUCTION

Inflammation is believed to contribute to the pathogenesis of Alzheimer's disease (AD)<sup>[1,2]</sup>. Complement protein cascades, cyclooxygenases (COX), oxygen free radical species, cytokines, chemokines, and other inflammatory factors such as acute phase proteins have been linked to the development and progression of AD<sup>[2,3]</sup>. Release of pro-inflammatory mediators by microglia, the main effector cells of inflammation within the central nervous system (CNS), could be sufficient to cause AD pathology independently of other pathological events, such as deposition of the amyloid  $\beta$  protein (A $\beta$ ) and neurofibrillary tangles (NFT)<sup>[4,5]</sup>. Elevated nitric oxide (NO) released by astrocytes and microglia during neurodegenerative disease is both necessary and sufficient to induce primary microglial phagocytosis of neurons and leads to neurotoxic effects resulting from perturbed mitochondrial respiration<sup>[6-8]</sup>. Down-regulation of inducible nitric oxide synthase (iNOS) in AD-model mice, and subsequent decrease in NO, has been associated with rescue of cognitive function, reduction in A $\beta$  and NFT load, decreased glial activation, and attenuated neuronal loss<sup>[9-11]</sup>. As dysregulated activation of microglia resulting from inflammatory insult has been closely associated with AD brain regions exhibiting extensive deterioration, A $\beta$  deposition, and markers of NO-mediated protein damage<sup>[8,12,13]</sup>, it has been suggested that reducing microglial activation may be an effective means of treating neurodegenerative diseases. Epidemiological studies indicate that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit microglial COX and their select immune functions, reduces the relative risk of developing AD<sup>[13,14]</sup>; however, several clinical trials have failed to identify protective activity of NSAIDs in AD patients<sup>[15-17]</sup>.

While not generally considered an anti-inflammatory drug, acetaminophen (paracetamol, N-acetylpara-aminophenol, APAP) has similar clinical indications to NSAIDs due to its analgesic and antipyretic properties<sup>[18]</sup>. Acetaminophen inhibits the synthesis of prostaglandin (PG) E<sub>2</sub>, PGF<sub>2</sub>, and thromboxane B<sub>2</sub> by lipopolysaccharide (LPS)-stimulated microglia, yet has no effect on the levels of pro-inflammatory mediators such as tumor necrosis factor (TNF)- $\alpha$  and NO<sup>[19,20]</sup>. These effects are attributed to the inhibition of COX enzymatic activity, as the expression of the enzymes involved in PG synthesis, including COX, is not affected nor does the concentration of the PG precursor, arachidonic acid, significantly vary with acetaminophen treatment<sup>[20]</sup>. Moreover, the inhibition of COX by acetaminophen has been shown to be more efficacious in microglia than in peripheral macrophages<sup>[20,21]</sup>. It has been suggested that the low levels of oxidants in the CNS potentiate the ability of acetaminophen to reduce the catalytically active oxidized form of COX to its inactive state<sup>[21]</sup>. It has, therefore, been proposed that acetaminophen may be a good agent for treating neuroinflammation in the CNS, without compromising peripheral PG levels<sup>[20]</sup>.

In addition to COX inhibition, the cannabinoid system has been suggested as the possible pharmacological target of acetaminophen. Antagonists of cannabinoid receptors inhibited the analgesic activity of acetaminophen by reducing the responsiveness of mice to nociceptive stimuli, attributable to the modulation of cytokine and NO pathways<sup>[22,23]</sup>. These studies suggest that the therapeutic activity of acetaminophen could be mediated, at least in part, through the effects of the parent drug or its metabolites on the cannabinoid system<sup>[24]</sup>. Studies on pharmacokinetics have indicated that acetaminophen undergoes extensive phase I and phase II metabolism prior to excretion. Initially, it was postulated that acetaminophen is metabolized by sulfotransferases (30%-44%) and uridine-5'-diphospho-glucuronosyltransferases (52%-57%) in the liver to inactive secondary compounds<sup>[25]</sup>. In 2005, however, an alternative metabolic pathway was described in which acetaminophen undergoes deacetylation to a lipid-soluble intermediate p-aminophenol, catalyzed to some extent by liver acylamidase and N-deacetylase<sup>[26-28]</sup>. Following distribution to the CNS, p-aminophenol is conjugated to arachidonic acid via fatty acid amide hydrolase (FAAH) to form the bioactive N-acylphenolamine, N-arachidonoylaminophenol (AM404)<sup>[27-29]</sup>. A single dose of acetaminophen commonly used to induce analgesia in rats (300 mg/kg body weight) leads to



**Figure 1.** Acetaminophen undergoes deacetylation in the liver (grey) by acylamidase and N-deacetylase to yield p-aminophenol. P-aminophenol is distributed through the cardiovascular system (white/grey) to the central nervous system (CNS) (white). In the CNS, p-aminophenol is conjugated to arachidonic acid by fatty acid amide hydrolase (FAAH) to yield AM404. FAAH also breaks down anandamide to arachidonic acid and ethanolamine. The catabolic activity of FAAH on anandamide is counterbalanced by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), which re-synthesizes anandamide from arachidonic acid and ethanolamine

approximately 7  $\mu\text{mol/L}$  p-aminophenol and 10  $\text{nmol/L}$  AM404 in the brain<sup>[27]</sup>. Figure 1 illustrates the metabolic pathways of acetaminophen from its deacetylation in the liver, to the formation of the bioactive N-acylphenolamine, AM404. The concomitant activity of FAAH in catabolizing the endocannabinoid anandamide (also known as N-arachidonylethanolamine, AEA) to its metabolites ethanolamine and arachidonic acid is also presented.

Originally reported as an endogenous cannabinoid reuptake inhibitor, AM404 has various roles in nociceptive and thermoregulatory pathways including potentiating the activity of anandamide through inhibition of FAAH, acting as an agonist of the transient receptor potential cation channel subfamily V member 1 (TRPV1), inhibiting enzymatic activity of the two COX isoforms (COX-1 and COX-2), and preventing NF- $\kappa$ B activation<sup>[27,30]</sup>. Upon the discovery that AM404 is a bioactive metabolite of acetaminophen, it was hypothesized that AM404 may be responsible for some of the pharmacological activities of acetaminophen *in vivo*. Several studies have since confirmed that AM404 contributes to the analgesic, antinociceptive, and anxiolytic activities of acetaminophen through its effects on cannabinoid receptor type 1 (CB1) and FAAH<sup>[22,31,32]</sup>. In mice, acetaminophen exerts an AM404-mediated anxiolytic effect that is dependent on CB1 signaling. Other studies have identified that the metabolites of acetaminophen, p-aminophenol or AM404, can indirectly activate CB1 receptors by inhibiting cellular uptake of the endogenous CB1 receptor agonist anandamide<sup>[32,33]</sup>. Additionally, FAAH inhibition has recently been described to initiate protective responses in neurodegenerative diseases, alleviating oxidative damage in the hippocampus and frontal

cortex<sup>[34]</sup>. In summary, some of the pharmacological activity of acetaminophen is mediated by its bioactive metabolites p-aminophenol and AM404. It is currently unknown whether p-aminophenol and AM404 have protective effects beyond COX inhibition that may indicate the use of acetaminophen as an effective means of treating neurodegenerative diseases. Due to resounding evidence that NO contributes directly to the pathogenesis of AD, this study evaluated the possible beneficial effects of acetaminophen and its metabolites in the neurodegenerative disease pathology as inhibitors of the release of NO from activated microglia.

## METHODS

### BV-2 cell culture

BV-2 cells were suspended at 0.2 million cells/mL in Dulbecco's Modified Eagle's Medium/F12 containing 100 U/mL penicillin, 100 µg/mL streptomycin and 5% calf bovine serum (F5) (all from Fisher Scientific, Ottawa, ON, Canada). Two mL of cell suspension were added to each well of a 24-well plate (Corning Inc., Corning, NY, USA) and incubated for 24 h at 37 °C in 5% CO<sub>2</sub> to allow for adherence. Cells were treated with various concentrations of URB597 or 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC) (0.02, 0.5, 2 µmol/L, Cayman Chemical Company, Ann Arbor, MI, USA), SR144528 (0.02, 0.5, 2 µmol/L, EMD Millipore, Etobicoke, ON, Canada), indomethacin (2, 20, 50 µmol/L, Sigma-Aldrich, Oakville, ON, Canada), or vehicle solution (0.1% dimethyl sulfoxide, DMSO) and incubated for 15 min at 37 °C in 5% CO<sub>2</sub>. BV-2 cells were subsequently treated with various concentrations (2, 20, 50 µmol/L) of acetaminophen (MP Biomedicals, Solon, OH, USA), p-aminophenol (Sigma-Aldrich), AM404 (Cayman), or vehicle solution (0.1% DMSO) and incubated for a further 15 min period under the same conditions. Subsequently, BV-2 cells were stimulated with LPS (0.5 µg/mL, Sigma-Aldrich) for 24 h to induce NO secretion. In our experiments, the specific inhibitors URB597, BCTC, and SR144528 were used at concentrations at least ten times higher than their reported IC<sub>50</sub> values, thus ensuring inhibition of their respective targets.

### Griess assay for nitrite detection

Secretion of NO by murine BV-2 cells was quantified indirectly by measuring the accumulation of its stable breakdown product, nitrite<sup>[35-38]</sup>. Briefly, 50 µL of cell culture supernatant from each well of a 24-well plate were transferred to a 96-well plate. 50 µL sodium nitrite solutions in F5 (0.1-40 µmol/L) were also added to the 96-well plate. An equal volume of Griess reagent, prepared immediately beforehand by combining 1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid (all from Sigma-Aldrich) was then added to each well and absorbance at 570 nm was measured. Absorbance values for test wells were normalized relative to control supernatants obtained from unstimulated BV-2 cells and nitrite concentration was calculated from the standard curve obtained by using solutions of sodium nitrite at different concentrations.

### Lactate dehydrogenase cytotoxicity assay

Cellular death results in the loss of cell membrane integrity and an uncontrolled release of intracellular components into the extracellular space, including the release of cytoplasmic lactate dehydrogenase (LDH). Activity of this enzyme can be quantified to measure the extent of cell death<sup>[39]</sup>. Briefly, 100 µL of cell-free supernatant from each well of a 24-well plate were transferred to a 96-well plate and 20 µL of iodonitrotetrazolium chloride (4 mg/mL, Sigma-Aldrich) were added to each well. Absorbance was measured at 490 nm. Next, a solution was prepared consisting of lactate (750 µg/mL), β-nicotinamide adenine dinucleotide (60 µg/mL), and diaphorase (55 µg/mL) (all from Sigma-Aldrich) in phosphate-buffered saline (PBS); 30 µL of the solution were added to each well and absorbance was measured at 490 nm following a 30-min incubation at 37 °C. Cell death was calculated as a percent relative to total LDH level measured in cultures of untreated cells lysed with 1% Triton X-100 (100% lysed cells).

### MTT cell viability assay

MTT is a water-soluble tetrazolium dye that is converted by the mitochondrial enzyme succinate dehydrogenase to an insoluble purple formazan in live cells. Spectrophotometric quantification of formazan dye present in the cells allows for determination of cell viability<sup>[40]</sup>. The MTT assay is more sensitive in detecting cytotoxicity when compared with the LDH assay; however, it may also incorrectly report mitochondrial dysfunction as cell death<sup>[41,42]</sup>. Therefore, the MTT and the LDH assays were used in parallel in this study. The MTT cell viability assay was conducted by, first, adding MTT (Sigma-Aldrich) to the cell cultures in a 24-well plate to reach a final concentration of 500 µg/mL. Plates were incubated for 1 h at 37 °C in 5% CO<sub>2</sub> to allow for cellular metabolism of the tetrazolium dye. Then 20% sodium dodecyl sulfate/50% N,N-dimethylformamide (both from Fisher Scientific) solution in milliQ water (EMD Millipore) was added 1:1 to the cell culture in each well to lyse the cells and solubilize the formazan crystals. Plates were incubated overnight at 37 °C until the crystals dissolved and absorbance of each sample was measured at 570 nm. Cellular viability was calculated as a percent relative to fully viable cells incubated in fresh cell culture media only.

### Statistical analysis

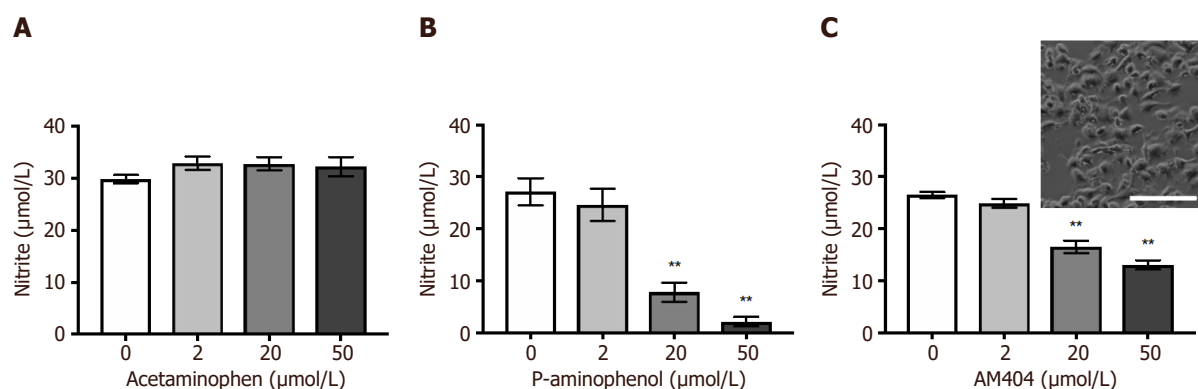
Data were analyzed with Prism V7.0c (Graphpad Software, Inc., USA). One-way and two-way analyses of variance (ANOVA) were performed, followed by Dunnett's *post hoc* test and Tukey's honestly-significant difference (HSD) test, respectively. Data are presented as means ± standard error of the mean (SEM). *P*-values less than 0.05 were considered statistically significant.

## RESULTS

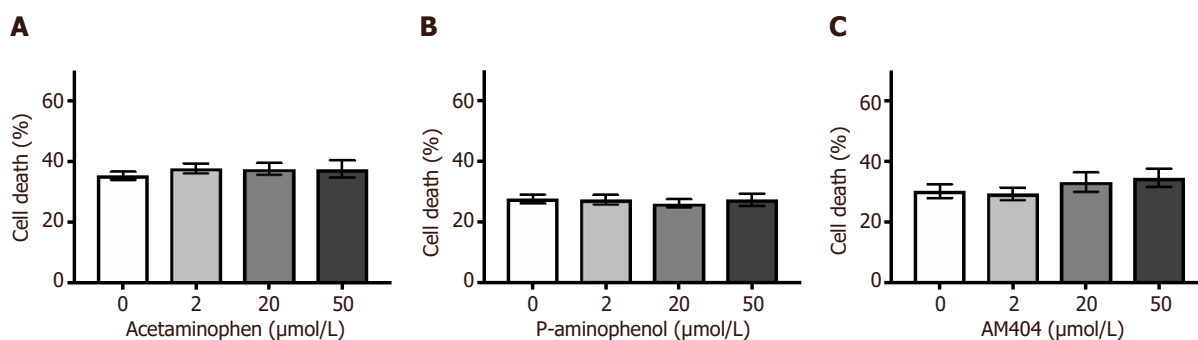
The aim of this study was to determine whether the inhibitory effects of acetaminophen on microglia-driven inflammation are mediated by the parent acetaminophen molecule or its metabolites, p-aminophenol and AM404. BV-2 microglial cells were used, which is a widely-accepted model of primary microglia<sup>[36]</sup>. Their activation was induced by LPS, which, similar to several endogenous pro-inflammatory molecules, triggers expression of inducible nitric oxide synthase (iNOS), leading to release of neurotoxic concentrations of NO by microglia<sup>[43-46]</sup>.

Clinically, acetaminophen is indicated for treatment of fever and pain. It has been reported to affect several microglial functions *in vitro*. In LPS-stimulated microglia, acetaminophen suppressed the synthesis of the inflammatory mediators PGE<sub>2</sub>, PGF<sub>2</sub>, and thromboxane B<sub>2</sub> by inhibiting the activity of COX; however, it had no effect on the levels of the pro-inflammatory mediators TNF-α and NO<sup>[19,20]</sup>. Data in [Figure 2A](#) confirm these observations by showing that at therapeutic as well as supratherapeutic concentrations acetaminophen had no significant effect on NO released by LPS-stimulated BV-2 microglia. Both metabolites of acetaminophen studied, p-aminophenol and AM404, at 20 and 50 µmol/L significantly inhibited this parameter of microglial activation [[Figure 2B and C](#)]. It is important to note that at the concentrations studied none of the three compounds reduced the viability of BV-2 cells, relative to vehicle-treated control cells, measured by the LDH [[Figure 3](#)] and MTT assays [[Figure 4](#)]. Stimulation of BV-2 cells by LPS causes significant increase in cell death (30%-40% cell death, [Figure 3](#)) and reduction in cell viability (50%-80% cell viability, [Figure 4](#)) in the absence of any of the compounds studied. Adding acetaminophen, p-aminophenol or AM404 did not lead to further enhancement of the LPS-induced cell death. Because nontoxic concentrations of inhibitors were used, it was concluded that the observed decreases in NO secretion by stimulated BV-2 microglia were caused by inhibition of specific signaling pathways.

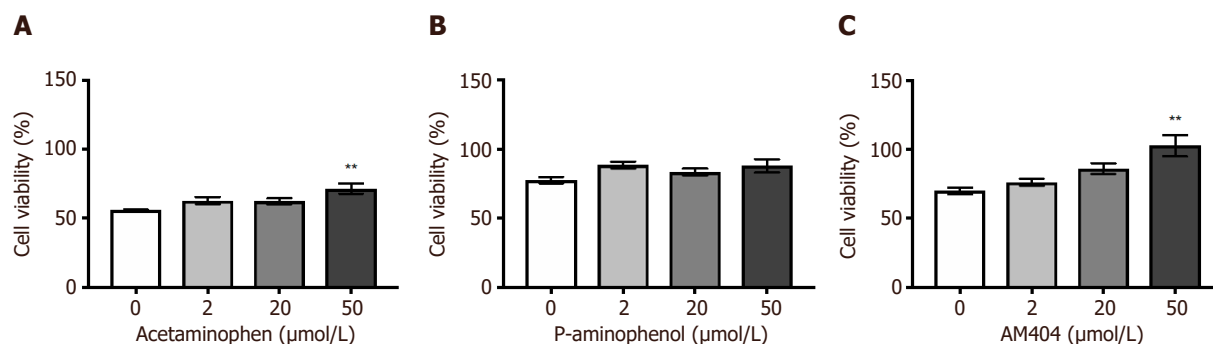
Previous reports have suggested that at least some of the clinical effects of acetaminophen could be due to its metabolism to AM404 or the deacetylated intermediate, p-aminophenol<sup>[47]</sup>. Following distribution to the CNS, p-aminophenol is conjugated to arachidonic acid through the catalytic activity of FAAH,



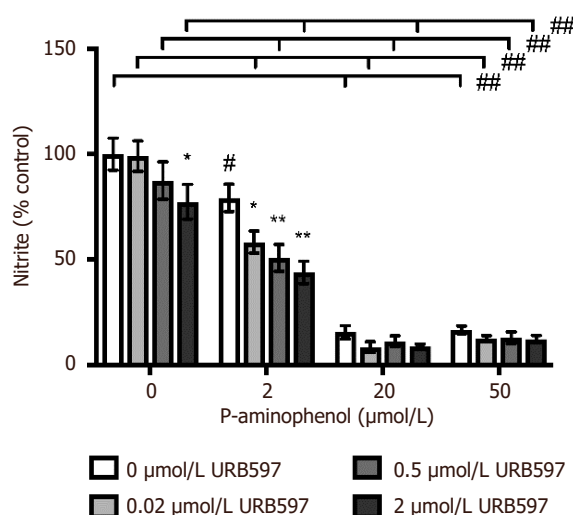
**Figure 2.** Effects of acetaminophen (A), p-aminophenol (B), and AM404 (C) on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated with various concentrations of drugs (2, 20, 50  $\mu\text{mol/L}$ ) for 15 min, then stimulated with LPS. Nitrite concentrations in BV-2 cell-free supernatants were measured after 24 h using the Griess assay. Data (means  $\pm$  SEM) from 3-5 independent experiments are presented. The effects of the treatments were assessed by the one-way analyses of variance (ANOVA), followed by Dunnett's *post hoc* test. \*\* $P < 0.01$ , significantly different from stimulated control cells incubated in the absence of drugs.  $F$  and  $P$  values for the main effects of one-way ANOVA are: (A)  $F = 1.09$ ,  $P = 0.41$ ; (B)  $F = 29.33$ ,  $P < 0.0001$ ; (C)  $F = 50.28$ ,  $P < 0.0001$ . Insert in (C) is a phase-contrast image of LPS-stimulated BV-2 microglia (scale bar = 200  $\mu\text{m}$ )



**Figure 3.** Effects of acetaminophen (A), p-aminophenol (B), and AM404 (C) on lipopolysaccharide (LPS)-induced cell death. BV-2 cells were treated with various concentrations of drugs (2, 20, 50  $\mu\text{mol/L}$ ) for 15 min, then stimulated with LPS. Following 24 h incubation, cell death was measured using the LDH assay. Data (mean  $\pm$  SEM) from 3-5 independent experiments are presented. The effects of the treatments were assessed by the one-way analyses of variance (ANOVA), followed by Dunnett's *post hoc* test. No significant differences were observed.  $F$  and  $P$  values for the main effects of one-way ANOVA are: (A)  $F = 0.31$ ,  $P = 0.82$ ; (B)  $F = 0.17$ ,  $P = 0.92$ ; (C)  $F = 0.84$ ,  $P = 0.48$



**Figure 4.** Effects of acetaminophen (A), p-aminophenol (B), and AM404 (C) on lipopolysaccharide (LPS)-induced cell viability. BV-2 cells were treated with various concentrations of drugs (2, 20, 50  $\mu\text{mol/L}$ ) for 15 min, then stimulated with LPS. Following 24 h incubation, cell viability was measured using the MTT assay. Data (mean  $\pm$  SEM) from 3-5 independent experiments are presented. The effects of the treatments were assessed by the one-way analyses of variance (ANOVA), followed by Dunnett's *post hoc* test. \*\* $P < 0.01$ , significantly different from stimulated control cells incubated in the absence of drugs.  $F$  and  $P$  values for the main effects of one-way ANOVA are: (A)  $F = 6.09$ ,  $P = 0.018$ ; (B)  $F = 2.51$ ,  $P = 0.066$ ; (C)  $F = 9.39$ ,  $P = 0.0001$



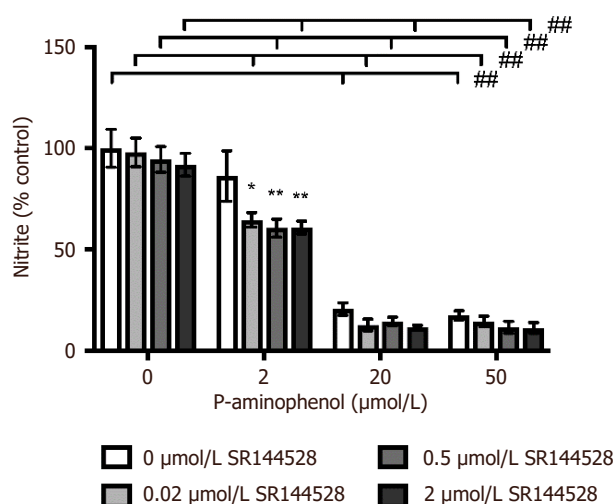
**Figure 5.** Effects of p-aminophenol alone, or in combination with the fatty acid amide hydrolase inhibitor URB597, on the lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of URB597 (0.02, 0.5, 2 μmol/L), then incubated with different concentrations (2, 20, 50 μmol/L) of p-aminophenol for a further 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from nine independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $13.2 \pm 1.0$  μmol/L. The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test. \* $P < 0.05$  and \*\* $P < 0.01$ , significantly different from stimulated cells incubated in the absence of URB597 (ANOVA  $F = 7.87$ ,  $P < 0.0001$ ); # $P < 0.05$  and ## $P < 0.01$ , significantly different from stimulated cells incubated in the absence of p-aminophenol (ANOVA  $F = 216.2$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 1.83$ ,  $P = 0.068$

forming AM404<sup>[27]</sup>. In the CNS, AM404 activates TRPV1 but inhibits COX-1 and COX-2 enzymatic activity, anandamide reuptake and metabolism, as well as the transcription factors NF-κB and nuclear factor of activated T-cells (NFAT)<sup>[23,30]</sup>. Due to these various pharmacological activities, AM404 has been implicated as the active substance responsible for the clinically beneficial effects of acetaminophen on nociception, mechanical allodynia, thermal hyperalgesia, as well as inflammatory processes resulting from the gene activation induced by the transcription factors NF-κB and NFAT. Specifically, the ability of AM404 to alleviate neuropathic pain in rodent models has been attributed to a reduction in NO and cytokine production<sup>[23]</sup>. In this study, we report that AM404 suppresses NO secretion by LPS-stimulated BV-2 cells [Figure 2C].

Since both p-aminophenol and AM404 effectively reduced NO release by stimulated BV-2 microglia, we hypothesized that FAAH present in BV-2 cells may allow p-aminophenol to exert its effect on microglial activation through its metabolism to AM404<sup>[48]</sup>. To test this hypothesis, BV-2 cells were incubated with various concentrations (0.02, 0.5, 2 μmol/L) of the specific FAAH inhibitor URB597 for 15 min prior to treatment with p-aminophenol. We rationalized that the inhibitory effect of p-aminophenol would be eliminated in the presence of URB597, if this metabolite were working through its conversion to AM404. As illustrated in Figure 5, p-aminophenol attenuated NO secretion from stimulated microglia in the presence of all concentrations of URB597 tested. Interestingly, URB597 itself showed an inhibitory effect on NO release in the absence of p-aminophenol and in the presence of 2 μmol/L p-aminophenol. These results indicate that, contrary to the initial hypothesis, p-aminophenol inhibits NO secretion through a pathway independent of its conversion to AM404 by FAAH. Moreover, p-aminophenol and URB597 may act synergistically to abate microglial activation.

It has been suggested that URB597 may function to attenuate microglial activation through the inhibition of FAAH<sup>[49]</sup>. In addition to catalyzing the conjugation of p-aminophenol and arachidonic acid, FAAH functions in the degradation of fatty acid amides such as the endocannabinoid anandamide<sup>[50]</sup>. In this

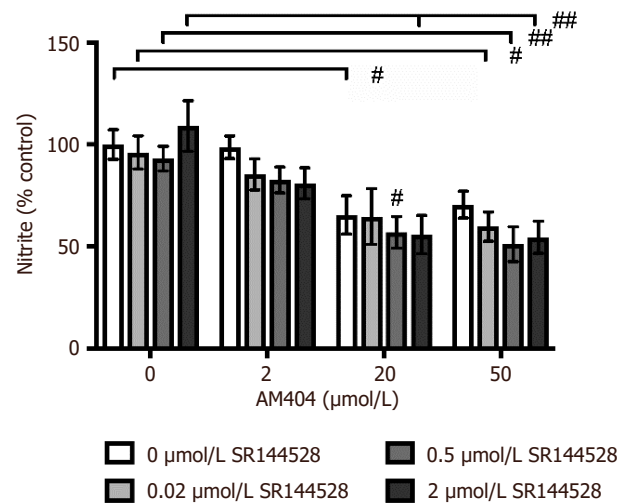




**Figure 6.** Effects of p-aminophenol alone or in combination with the selective CB2 receptor antagonist SR144528 on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of SR144528 (0.02, 0.5, 2 μmol/L), then incubated with different concentrations (2, 20, 50 μmol/L) of p-aminophenol for a 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from four independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $19.6 \pm 1.8$  μmol/L. The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test. \* $P < 0.05$  and \*\* $P < 0.01$ , significantly different from stimulated cells incubated in the absence of SR144528 (ANOVA  $F = 4.17$ ,  $P = 0.011$ ); ### $P < 0.01$ , significantly different from stimulated cells incubated in the absence of p-aminophenol (ANOVA  $F = 232.2$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 0.79$ ,  $P = 0.62$ .

capacity, URB597 indirectly activates cannabinoid receptors by potentiating the effect of bioavailable anandamide. Similarly, it has been identified that AM404 inhibits anandamide reuptake and degradation through mechanisms which are currently unknown<sup>[51,52]</sup>. Accordingly, it has been reported that URB597 and AM404 have similar effects on LPS-induced inflammation in rats that are CB1 and CB2 receptor dependent<sup>[53]</sup>. These observations are consistent with our data showing that URB597 on its own [Figure 5], similar to AM404 [Figure 2C], inhibits nitrite secretion by BV-2 microglia.

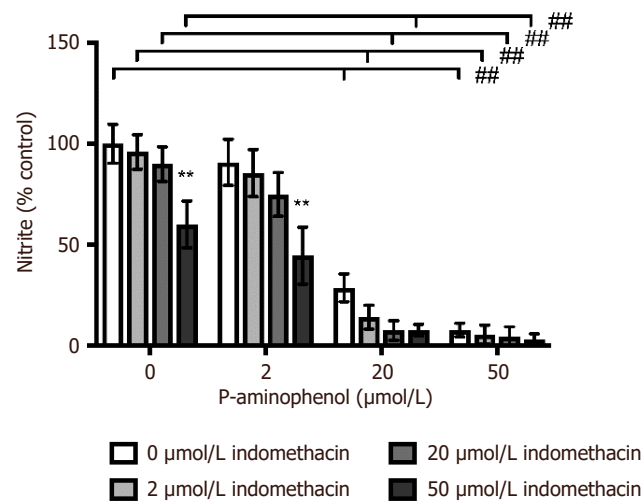
Next, we investigated the role of cannabinoid receptor signaling in mediating the effect of p-aminophenol and AM404 on NO secretion by LPS-stimulated microglia. Controversy exists surrounding the ability of AM404 to directly bind and activate CB1 receptors; however, it is widely agreed that AM404 attenuates the reuptake and degradation of the endogenous cannabinoid, anandamide, thereby potentiating its agonistic effect at CB1 and CB2 receptors<sup>[23]</sup>. Moreover, it has been suggested that AM404-mediated activation of TRPV1 is sufficient to induce the synthesis of additional anandamide, further increasing signaling through both CB1 and CB2 receptors<sup>[54]</sup>. It has been previously reported that signaling through CB1 receptors has neurotoxic and psychoactive effects, while signaling through CB2 receptors reduces the toxicity of the LPS-stimulated microglia<sup>[55]</sup>. As BV-2 cells express functionally active CB2 receptors, we determined whether the effect of p-aminophenol and AM404 on microglial activation was mediated by CB2 receptor signaling<sup>[56-58]</sup>. Microglia were incubated with various concentrations of the selective CB2 receptor antagonist SR144528 (0.02, 0.5, 2 μmol/L) for 15 min prior to treatment with p-aminophenol or AM404. Figures 6 and 7 indicate that CB2 receptor blockade did not reduce the secretion of NO from LPS-stimulated BV-2 cells in the absence of p-aminophenol and AM404. SR144528 also did not attenuate the inhibitory effects of p-aminophenol [Figure 6] or AM404 [Figure 7]; however, treatment with SR144528 did inhibit NO secretion in the presence of 2 μmol/L p-aminophenol [Figure 6]. These data indicate that neither AM404 nor p-aminophenol exert their effect on NO production by BV-2 cells through engaging the CB2 receptor and that CB2 receptor blockade may enhance the inhibitory effects of p-aminophenol on microglial activation.



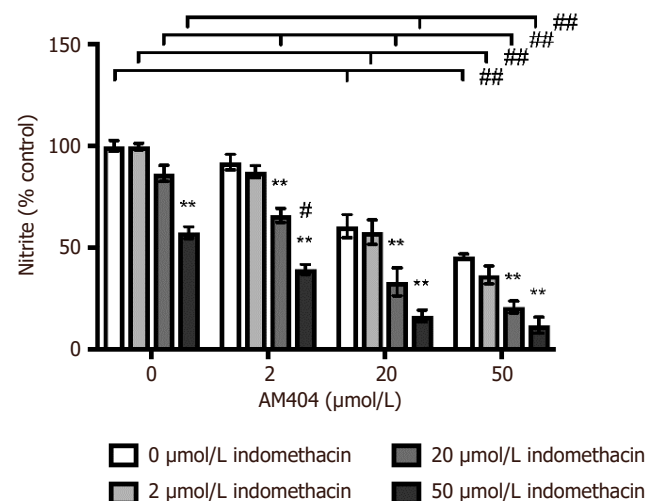
**Figure 7.** Effects of AM404 alone or in combination with the selective CB2 receptor antagonist SR144528 on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of SR144528 (0.02, 0.5, 2 μmol/L), then incubated with different concentrations (2, 20, 50 μmol/L) of AM404 for a further 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from four independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $18.9 \pm 1.4$  μmol/L. The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test. No significant differences were observed for stimulated cells incubated in the absence of SR144528 (ANOVA  $F = 1.56$ ,  $P = 0.21$ ). # $P < 0.05$  and ## $P < 0.01$ , significantly different from stimulated cells incubated in the absence of AM404 (ANOVA  $F = 22.23$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 0.45$ ,  $P = 0.90$ .

Subsequently, we studied whether p-aminophenol and AM404 suppress NO release from activated microglia through inhibition of COX-1 and COX-2. Involvement of COX was investigated because previous studies have demonstrated that AM404 modulates NO release as well as inhibits purified COX isoforms 1 and 2<sup>[23,27]</sup>. Furthermore, BV-2 cells express both isoforms of COX and these enzymes participate in inflammatory processes involving the AM404 precursor, arachidonic acid<sup>[59]</sup>. BV-2 cells were exposed for 15 min to various concentrations (2, 20, 50 μmol/L) of the nonselective COX inhibitor indomethacin prior to treatment with p-aminophenol or AM404 [Figures 8 and 9]. Previous reports have indicated that indomethacin is effective at inhibiting NO production by LPS-stimulated microglia *in vitro*<sup>[60]</sup>. We hypothesized that if p-aminophenol or AM404 lost their ability to inhibit NO release by stimulated microglia in the presence of indomethacin, it could be concluded that they exert their effect through COX inhibition. Consistent with the previous studies, indomethacin on its own significantly reduced NO production by stimulated BV-2 cells<sup>[60]</sup>. Both p-aminophenol and AM404 were able to attenuate NO secretion by stimulated microglia in the presence of indomethacin at all concentrations used. Notably, p-aminophenol and AM404 caused a greater reduction in NO secretion than indomethacin. While we cannot completely exclude COX inhibition as one of the mechanisms of p-aminophenol or AM404 inhibitory activity, it appears not to be the primary mechanism by which these compounds reduce microglial activation. It is important to note that, unlike other inhibitors used, indomethacin at 50 μmol/L was significantly toxic to BV-2 cells according to the LDH and MTT cell viability assays (data not shown), which could also be partially responsible for its inhibitory effect on NO production at this high concentration.

After determining that p-aminophenol and AM404 attenuate microglial activation independently of COX inhibition, we explored the effects of these compounds on TRPV1 signaling. TRPV1 has been previously identified as a molecular target of AM404. Signaling through this receptor may contribute to the antiallodynic and antihyperalgesic effects of AM404, which are mediated by the NO pathway<sup>[23]</sup>. As such, we hypothesized that p-aminophenol and AM404 may reduce NO secretion from stimulated BV-2 microglia by interacting with TRPV1<sup>[61]</sup>. BV-2 cells were treated for 15 min with various concentrations

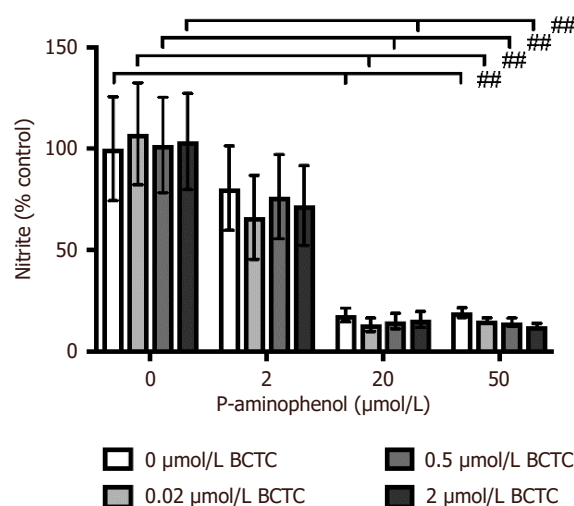


**Figure 8.** Effects of p-aminophenol alone or in combination with the non-selective cyclooxygenase inhibitor indomethacin on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of indomethacin (2, 20, 50  $\mu\text{mol/L}$ ), then incubated with different concentrations (2, 20, 50  $\mu\text{mol/L}$ ) of p-aminophenol for a further 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from four independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $27.1 \pm 2.6 \mu\text{mol/L}$ . The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test.  $**P < 0.01$ , significantly different from stimulated cells incubated in the absence of indomethacin (ANOVA  $F = 8.05$ ,  $P = 0.0002$ );  $##P < 0.01$ , significantly different from stimulated cells incubated in the absence of p-aminophenol (ANOVA  $F = 95.51$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 1.34$ ,  $P = 0.24$ .

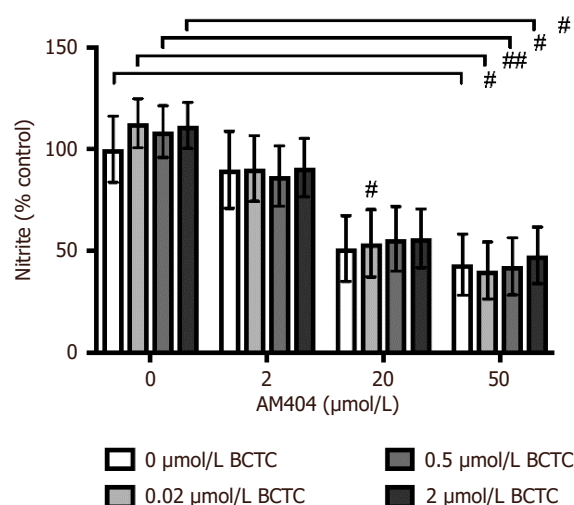


**Figure 9.** Effects of AM404 alone or in combination with the non-selective cyclooxygenase inhibitor indomethacin on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of indomethacin (2, 20, 50  $\mu\text{mol/L}$ ), then incubated with different concentrations (2, 20, 50  $\mu\text{mol/L}$ ) of AM404 for a further 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from four independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $26.6 \pm 0.7 \mu\text{mol/L}$ . The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test.  $**P < 0.01$ , significantly different from stimulated cells incubated in the absence of indomethacin (ANOVA  $F = 102.3$ ,  $P < 0.0001$ );  $*P < 0.05$  and  $##P < 0.01$ , significantly different from stimulated cells incubated in the absence of AM404 (ANOVA  $F = 179$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 1.79$ ,  $P = 0.10$ .

(0.02, 0.5, 2  $\mu\text{mol/L}$ ) of the selective TRPV1 antagonist BCTC to block signaling through this receptor prior to the addition of p-aminophenol or AM404. If BCTC reduces the inhibitory activity of p-aminophenol or AM404 on NO secretion from stimulated microglia, it could be concluded that these metabolites of acetaminophen exert their effect through TRPV1 signaling. Figures 10 and 11 illustrate that both



**Figure 10.** Effects of p-aminophenol alone or in combination with the TRPV1 antagonist BCTC on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of BCTC (0.02, 0.5, 2 μmol/L), then incubated with different concentrations (2, 20, 50 μmol/L) of p-aminophenol for a further 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from eight independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $9.4 \pm 2.4$  μmol/L. The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test. No significant differences were observed for stimulated cells incubated in the absence of BCTC (ANOVA  $F = 0.049$ ,  $P = 0.99$ ).  $^{##}P < 0.01$ , significantly different from stimulated cells incubated in the absence of p-aminophenol (ANOVA  $F = 29.65$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 0.060$ ,  $P = 0.99$ . BCTC: 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide



**Figure 11.** Effects of AM404 alone or in combination with the TRPV1 antagonist BCTC on lipopolysaccharide (LPS)-induced nitric oxide release. BV-2 cells were treated for 15 min with various concentrations of BCTC (0.02, 0.5, 2 μmol/L), then incubated with different concentrations (2, 20, 50 μmol/L) of AM404 for a further 15 min period, before being stimulated with LPS. After 24 h, nitrite concentrations in the BV-2 cell-free supernatants were measured using the Griess assay. Data from five independent experiments are normalized against nitrite concentration in samples stimulated in the absence of inhibitors. Nitrite concentration in these samples was  $16.5 \pm 2.7$  μmol/L. The effect of treatments was assessed by the two-way analyses of variance (ANOVA), followed by Tukey's *post hoc* test. No significant differences were observed for stimulated cells incubated in the absence of BCTC (ANOVA  $F = 0.096$ ,  $P = 0.96$ ).  $^{*}P < 0.05$  and  $^{##}P < 0.01$ , significantly different from stimulated cells incubated in the absence of AM404 (ANOVA  $F = 16.45$ ,  $P < 0.0001$ ). ANOVA interaction  $F = 0.047$ ,  $P = 0.99$ . BCTC: 4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide

p-aminophenol and AM404 attenuated NO secretion from activated BV-2 cells in the presence of BCTC at all concentrations used. BCTC on its own did not have a significant effect on the NO secretion by stimulated BV-2 microglia [Figures 10 and 11] or their viability (data not shown).

## DISCUSSION

Our data indicate that the inhibitory effect of p-aminophenol and AM404 on NO secretion by stimulated BV-2 microglia is not caused by COX inhibition, or interaction with CB2 or TRPV1 receptors. Furthermore, enzymatic conversion of p-aminophenol to AM404 by FAAH is not required for its pharmacological activity in BV-2 cell cultures. Stimulation of microglia with LPS has been shown to induce the expression of several genes that are regulated by mitogen-activated protein kinases (MAPKs) and NF- $\kappa$ B, including such cytokines as TNF- $\alpha$ , interleukin (IL)-1 $\beta$ , interferon (IFN)- $\gamma$ , and their receptors, as well as the stress proteins superoxide dismutase (SOD) 2, COX-2, and thioredoxin interacting protein<sup>[62,63]</sup>. Moreover, it has already been documented that stimulation of BV-2 murine microglia with LPS induces the expression of iNOS in a MAPK- and NF- $\kappa$ B-dependent manner<sup>[64-66]</sup>. Previous studies have also determined that AM404 prevents the activation of transcription factors NFAT and NF- $\kappa$ B but preserves extracellular signal-regulated kinases (ERK)1/2 MAPK signaling *in vivo*<sup>[30,67]</sup>. Moreover, as NFAT is not directly activated by LPS, the only signaling pathway in this study that is both inhibited by AM404 and activated in microglia following stimulation with LPS is NF- $\kappa$ B<sup>[30,68]</sup>. AM404 has been shown to attenuate NF- $\kappa$ B activation by inhibiting the phosphorylation, and subsequent degradation, of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I $\kappa$ B $\alpha$ )<sup>[30]</sup>. Intact I $\kappa$ B $\alpha$  sequesters NF- $\kappa$ B in the cytoplasm by interfering with the function of the nuclear localization signal, thereby blocking NF- $\kappa$ B gene activation<sup>[69]</sup>. Considering that NF- $\kappa$ B activity is directly linked to the LPS-induced expression of iNOS and secretion of NO, it can be concluded that AM404 most likely modulates the secretion of NO from LPS-stimulated microglia through its inhibitory action on the NF- $\kappa$ B pathway<sup>[70,71]</sup>. This conclusion is consistent with previous studies where AM404 was demonstrated to block the overexpression of iNOS in models of neuropathic pain, supporting a mechanism of AM404 action where it inhibits NO secretion at the transcription level<sup>[23,72]</sup>.

## DECLARATIONS

### Authors' contributions

Conceived the study and wrote the manuscript: Slattery WT, Klegeris A

Conducted experiments and analyzed the data: Slattery WT

### Data source and availability

Data in this study were obtained by experimentation and are original. All primary data used to construct the summary figures are available by contacting the authors of this study.

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### Conflicts of interest

The authors declare that they have no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

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Commentary

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# Multiple sclerosis: depression and disability are globally shared issues of concern

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While multiple sclerosis (MS) is more common among Caucasian populations, its prevalence is increasingly noted in ethnic groups that had previously been considered to have a low frequency of the disease. Epidemiologic studies indicate a considerable augmentation of MS presence in areas such the Middle East<sup>[1]</sup> and Latin American countries<sup>[2]</sup>. Along with the general clinical effects of this complex neurological disorder is the impact exerted by some of its comorbid neuropsychiatric manifestations particularly depression, described as affecting three times higher MS patients than the general population<sup>[3]</sup>. Literature and general perceptions had suggested clinical depression, although chronic in most MS cases, was unrelated to disability progression or disease course<sup>[4,5]</sup>.

A Saudi Arabia study<sup>[6]</sup> published in *Neuroimmunology and Neuroinflammation*, acquired data from all geographic regions of the Kingdom involving 598 MS patients, and finding that 97.7% had some degrees of depression as assessed with the patient health questionnaire, a reliable and valid measure of depression severity. In this study, most had mild-to-moderate depression. The Saudi Arabian investigators correlated these data with scores obtained from disability quantified with the patient determined disease steps which has a strong correlation with the scores derived from the expanded disability status scale, the cardinal marker of neurological disability in MS. There was a significant association between patients' level of neurological disability and severity of depression ( $P < 0.001$ ). Interestingly, none of the patients reported absence of depression had a moderate or severe disability while those with moderately severe or severe depression had the highest percentages of severe disability. Aspects that escaped the authors explanation was the fact that severe depression was more prominent in patients residing in the northern areas of the country and that 13% of the subjects were foreigners, their country origin was not reported. Saudi Arabia has a medium to high MS prevalence ( $\geq 40/100,000$ )<sup>[7]</sup> has substantial immigrant population from nearby



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countries (and most recently refugees from Syria and Yemen), Asian expatriates and many westerners employed in professional and technical jobs. The reported phenomenon of people moving from a high-risk to a low-risk area carry the MS risk with them if the migration took place after age 15 continues to be discussed<sup>[8]</sup> but these epidemiologic aspects deserve further study in Saudi Arabia. Recent attention has been placed on the sociological and/or biological behavior of immigrants with MS moving from a lower to a higher risk country<sup>[9]</sup>. In this series, as it is universally observed, women's prevalence was higher (64.2%) with a female: male ratio of about 2:1. Other findings of note extracted from the data of the Saudi Arabian study are the higher prevalence of depression in young individuals ages 26-35 years ( $P = 0.001$ ) and with a higher education ( $P = 0.001$ ), but predominant as well in people with the lowest income (< 3000 monthly SR, equivalent to 800 USD).

The Saudi Arabian study provides the first-time evidence of a direct and significant relationship between depression and neurological disability in MS patients. In this sample, all the patients were being treated with a disease modifying therapy. The association of depression with the medication class remains unclear although the only two cases receiving Alemtuzumab (Lemtrada®) scored with severe depression and the highest percentages of moderately severe and severe depression were noted with  $\beta$  interferons 1-a (Avonex®; Rebif®) and 1-b (Betaferon®), as well as the oral product Dimethyl-fumarate (Tecfidera®). The presence of depression with its tremendous variety of emotional states in the majority of people with MS, is a factor requiring early identification and appropriate management in view of the impact to a person's quality of life and it is associated with progression of disease as well as neurological disability.

## DECLARATIONS

### Authors' contributions

The author contributed solely to the paper.

### Financial support and sponsorship

None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

### Copyright

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Original Article

Open Access



# Precentral gyrus abnormal connectivity in male and female patients with schizophrenia

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## Abstract

**Aim:** Dysfunction of the precentral gyrus plays a role in the impairments of voluntary movement associated with schizophrenia and it has significantly reduced functional activity in patients with schizophrenia. The aim of this study was to demonstrate the precentral gyrus alteration and its abnormal connectivity in schizophrenia.

**Methods:** The region of interest-based analysis method was used to investigate the precentral gyrus connectivity alteration in schizophrenia. The resting-state functional magnetic resonance imaging data of healthy control subjects and patients with schizophrenia (Centers of Biomedical Research Excellence data set) was used to examine the aberrant functional brain connectome in schizophrenia. This data set contains raw anatomical and functional magnetic resonance data from 72 patients with schizophrenia and 75 healthy controls, ranging in age from 18 to 65 years old.

**Results:** Our results show precentral gyrus has abnormal communication with thalamus, hippocampus, parahippocampal gyrus, posterior division of supramarginal gyrus and medial prefrontal cortex ( $pFDR = 0.05$ ). This information is expected to provide a better understanding of altered functional connectivity of the precentral gyrus in the male and female patients with schizophrenia.

**Conclusion:** Collectively, these findings support the hypothesis that precentral gyrus has an abnormal connectivity in schizophrenia and this alteration is not the same in the male and female patients with schizophrenia.

**Keywords:** Schizophrenia, functional magnetic resonance imaging, functional connectivity, resting-state, precentral gyrus



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## INTRODUCTION

Schizophrenia is a severe mental disorder, affecting ~1% of the population<sup>[1,2]</sup>. Recent studies have shown dysfunction of the precentral gyrus (PreCG) has long been thought to play a role in the impairments of voluntary movement associated with Schizophrenia and it has significantly reduced functional activity (FC) in patients with schizophrenia<sup>[3,4]</sup>. Also, schizophrenia is associated with volume deficits in PreCG<sup>[5]</sup>. Another research indicates the patients with schizophrenia showed lower activation in left PreCG than right PreCG<sup>[6]</sup> and a regional homogeneity (ReHo) study showed decreased ReHo in right precentral gyrus<sup>[7]</sup>. In this research, we examined the PreCG functional connectivity impairment in patients with schizophrenia using ROI based analysis<sup>[8]</sup>. We used the Center for Biomedical Research Excellence data set (COBRE)<sup>[9]</sup> to demonstrate how the functional connectivity of the PreCG with the rest of the brain regions changes in schizophrenia. In conjunction with previous studies<sup>[10-13]</sup>, our results indicate the PreCG has abnormal connectivity with brain regions like Thalamus and Hippocampus, but these impairments are not similar in two hemispheres. We also analyzed the functional connectivity differences between male and female patients with schizophrenia and showed the regions like Thalamus are more affected in the female patients with schizophrenia.

## METHODS

### Data

Two groups of subjects from the COBRE data set are used to examine the aberrant functional brain connectome in schizophrenia. COBRE data set contains raw anatomical and functional MRI data from patients with schizophrenia and healthy controls. Some papers that by the COBRE group published on this data<sup>[14-16]</sup>. This data set is available on and contains raw anatomical and functional MR data from patients with Schizophrenia and healthy controls, ranging in age from 18 to 65 years old. Resting fMRI, anatomical MRI, phenotype data for every participant including gender, age, handedness and diagnostic information are released<sup>[17]</sup>. More details about the COBRE data set can be found in the [Table 1](#).

### Analysis

Different preprocessing methods like realignment<sup>[18]</sup>, coregistering, normalization are applied to the structural and functional data. The functional volumes are coregistered with the region of interest and structural volumes<sup>[19,20]</sup>. Regions of interest and all the Brodmann areas defined by the Talairach daemon assigned to all subjects. By segmentation of structural image for each subject, grey matter, white matter and cerebrospinal fluid (CSF) mask generated. Here, the time series of interest are numbers of PCA components.

### First and second level of covariates

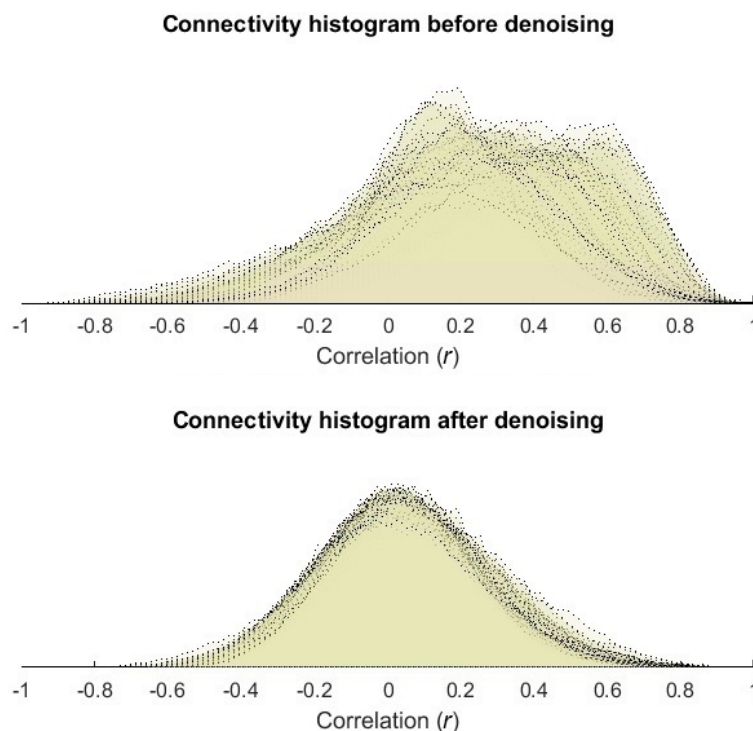
In this step, the realignment parameters in Blood-Oxygen-Level-Dependent (BOLD) model is defined (the first level covariate), then in the second level covariate, the group level regressor is performed. We categorized the data input data into 4 groups: females and males healthy control, females and males patients with schizophrenia. After defining the experimental data, the functional data is imported, then the structural data segmented to define the grey matter, white matter, the cerebrospinal fluid region of interest. By performing principal component analysis (PCA) on within region of interests the ROIs time series is extracted.

### Data preprocessing

Before analyzing the data, we need to explore and remove the confound. The different source of possible confounds like cerebrospinal fluid and white matter signal and within-subject covariate (realignment parameters) are considered. We chose the 5-dimension numbers of temporal components are being used. Similarly, numbers of dimension for the white matter was 5 and the derivative order was 0 and the histogram

**Table 1. Demographic characteristics of subjects in COBRE data**

	<b>N</b>	<b>Age (SD)</b>	<b>Female (%)</b>
Patients with schizophrenia	72	38.16 (13.89)	0.19
Healthy control	74	35.82 (11.58)	0.31

**Figure 1.** Connectivity histogram before and after denoising

plot  $r$  value before and after confounds removal and the band-pass filter is set to (0.008, 0.09). Here the histograms  $r$ -square to identify outlier subject and the quality control is computed for every subject. [Figure 1](#) shows the connectivity histogram before and after denoising.

The functional connectivity (CONN) and Statistical Parametric Mapping (SPM) toolboxes<sup>[21]</sup> used for spatially preprocessing (realignment, coregistering, normalization) of structural and functional data of functional data are done using SPM toolbox. The functional volumes are coregistered with the region of interest and structural volumes. ROI-to-ROI correlational analysis was carried out by CONN toolbox and SPM8. The preprocessing of the functional images considered of band-pass filtering of 0.008-0.09 Hz, motion correction, registration to structural images and spatial normalization to the MNI template. Then to reduce the physiological noise source, a Component-Based Noise Correction Method (CompCor) has been used<sup>[22]</sup>. CompCor can be used for the reduction of noise in both blood oxygenation level dependent and perfusion-based functional magnetic resonance imaging data. False discovery rate correction is used for multiple hypothesis testing. Number of PCA components to be extracted for each ROI is set to one. It means the time-series of interest is defined as the average BOLD activation within the ROI voxels, but it's possible to define it as the principal eigenvariates of the time-series within the ROI voxels. Regions of interest and all the Brodmann areas defined by Talairach daemon assigned to all subjects. By segmentation of structural image for each subject, grey matter, white matter, and CSF masks were generated. Bivariate correlation is used as a functional connectivity measure between two areas. General linear model (GLM)<sup>[23]</sup> used for comparison of connectivity results between genders and between control and Schizophrenia patients.

**Table 2. The connectivity contrast values between the PreCG right and the targets ( $P = 0.05$ ) (the complete list of the effects size is provided in the supplementary data)**

Analysis Unit	Statistic	p-unc	p-FDR
Seed intensity = 18.77, size = 5	PreCG r	$F(30)(113) = 3.17$	0.0000
PreCG r -Hippocampus l	$T(142) = 4.16$	0.0001	0.0074
PreCG r -Thalamus l	$T(142) = -3.92$	0.0001	0.0093
PreCG r -pPaHC l	$T(142) = 3.62$	0.0004	0.0151
PreCG r -Thalamus r	$T(142) = -3.54$	0.0005	0.0151
PreCG r -pSMG r	$T(142) = -3.53$	0.0006	0.0151

### ROI based analysis

We investigated the hypothesis of connectivity difference in schizophrenia and used ROI analyzing for the PreCG region of the brain. Two-sample *t*-test analyzes computed via SPM8<sup>[24]</sup> to compare the connectivity results of patients *vs.* controls and male patient *vs.* women patient to compare the connectivity across two groups. Connectivity values (Fisher-transformed correlation coefficients) between the seed and the identified ROI was extracted from the connectivity map. The different source of interest can be defined for analyzing.

### First level voxel-based analysis

For a subject or condition, it is possible to perform voxel-to-voxel analyzing that applies the matrix of voxel-to-voxel connectivity values and there is no need for the prior region of interest or seed analysis. In this method, we can investigate whole-brain connectivity. The voxel-based analyzing can be on connectivity pattern (principal component analysis) between a voxel and the rest of the brain (MVPA). Another voxel-based measure is available in CONN toolboxes is indexed that calculates the average local connectivity between each voxel and its neighbors (integrated local correlation)<sup>[25]</sup> or instead of average, the spatial asymmetry of the local connectivity can be used (radial correlation contrast)<sup>[26]</sup>. Also, instead of local connectivity, global connectivity pattern between a voxel and the rest of the brain can be used (e.g., radial similarity contrast)<sup>[27]</sup>. More details about measuring the index can be found in<sup>[28]</sup>.

### Second level analysis

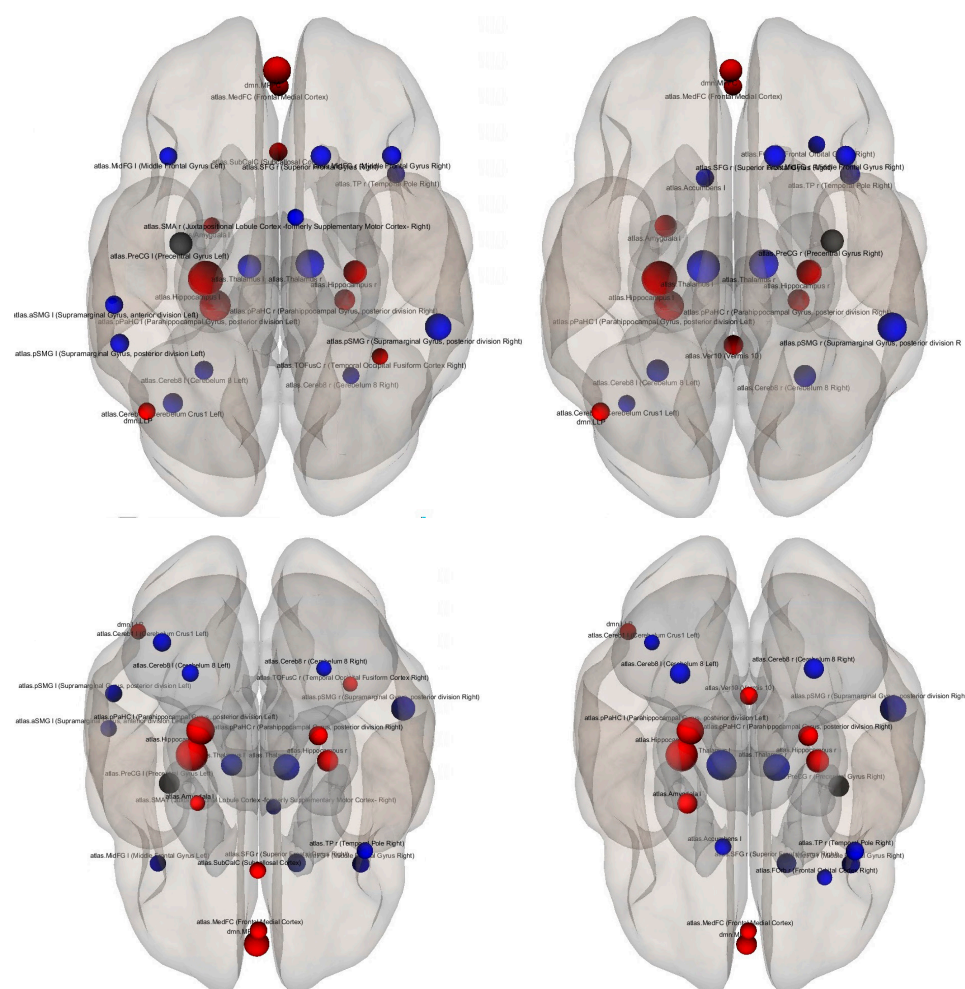
In the second level analysis step, the between-subject contrast can be considered (e.g., to compare different groups like male *vs.* female to see main effects in the connectivity within each group). In the ROI-to-ROI analyze, the first-level connectivity-measure matrix is used and the results can be a threshold at the desired *P*-value threshold. In this step by graph theoretical analyzing method provides the network measures like efficiency, centrality, and cost/degree to test the between-subject contrast.

## RESULTS

The connectivity contrast values between the PreCG right and the targets show the PreCG has abnormal communication with thalamus, hippocampus, pPaHC, pSMG and mPFC ( $P = 0.05$ ). Figure 2 shows ROI-level results for PreCG left and right PreCG. Effects size for each region is shown by the dot size. We compared the functional connectivity difference between the left and right PreCG between healthy controls and schizophrenia patients. Results show that the connectivity of the left Hippocampus with both left and right PreCG decreased in schizophrenia [Figure 3]. More details about the connectivity impairment of PreCG and the rest of the brain is demonstrated in the [Tables 2 and 3]. We also compared schizophrenia affects the PreCG in the male and female patients [Figures 4 and 5].

## DISCUSSION

The present study supports the notion of the functional connectivity abnormal connectivity of the precentral gyrus in schizophrenia<sup>[1,2,8-11]</sup>. We have shown that PreCG alteration and its abnormal connectivity are not the



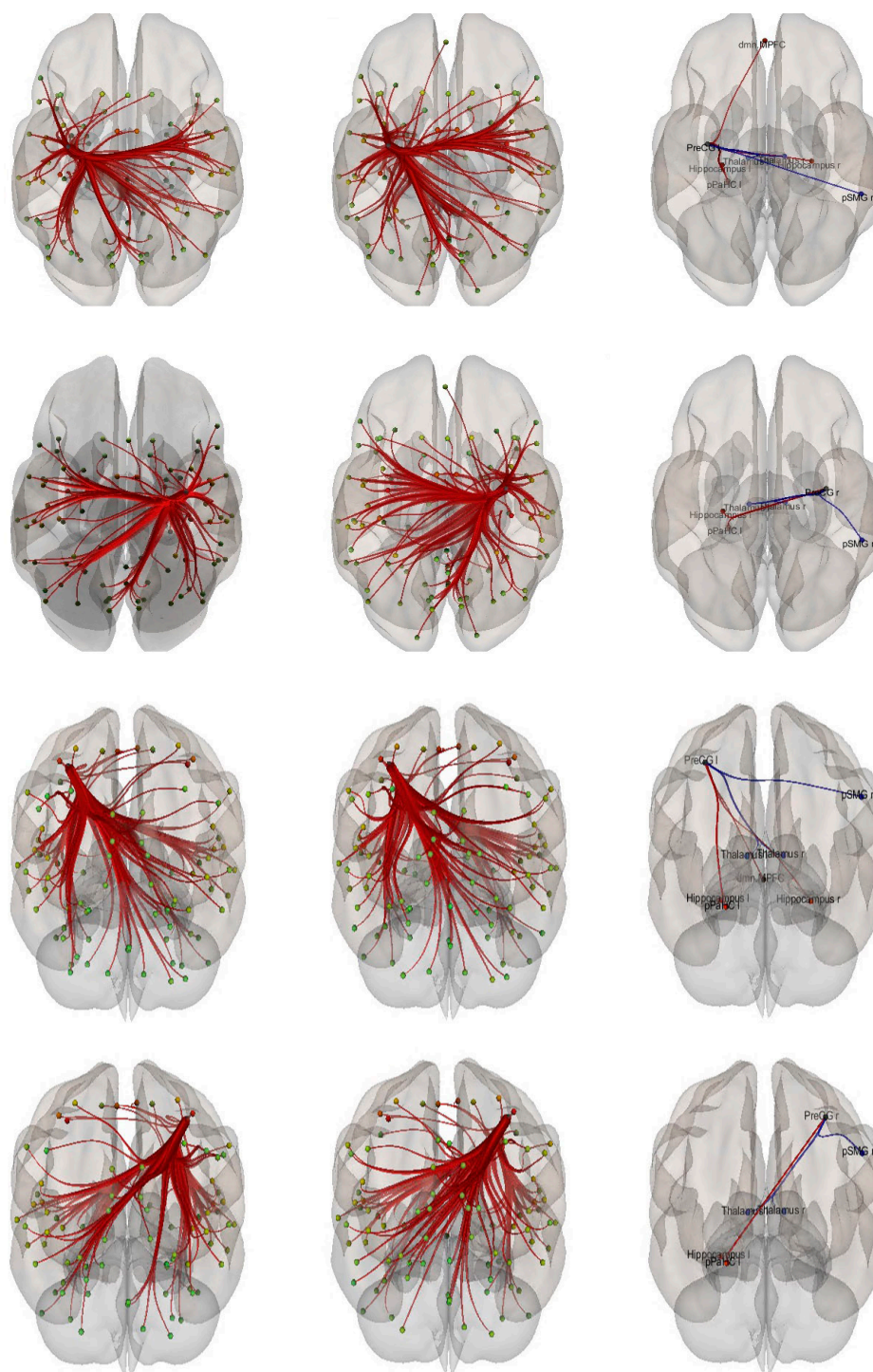
**Figure 2.** The superior (top) and the posterior views of the ROI-level results for each target ROI. The selected sources (precentral gyrus left and right) are shown by black dots and the targets are blue (control > schizophrenia) and red (control < schizophrenia). Effects size for each region is shown by the dot size

**Table 3. The connectivity contrast values between the PerCG left and the targets ( $P = 0.05$ ) (the complete list of the effects size is provided in the supplementary data)**

Analysis Unit	Statistic	p-unc	pFDR
Seed intensity = 25.55, size= 7	PreCG l	F(30)(113) = 3.41	0.0000
PreCG l -Hippocampus l	T(142) = 4.47	0.0000	0.0022
PreCG l -pPaHC l	T(142) = 4.02	0.0001	0.0063
PreCG l -Thalamus r	T(142) = -3.72	0.0003	0.0128
PreCG l -dmn.MPFC	T(142) = 3.64	0.0004	0.0131
PreCG l -pSMG r	T(142) = -3.48	0.0007	0.0179
PreCG l -Hippocampus r	T(142) = 3.12	0.0022	0.0450
PreCG l -Thalamus l	T(142) = -3.10	0.0023	0.0450

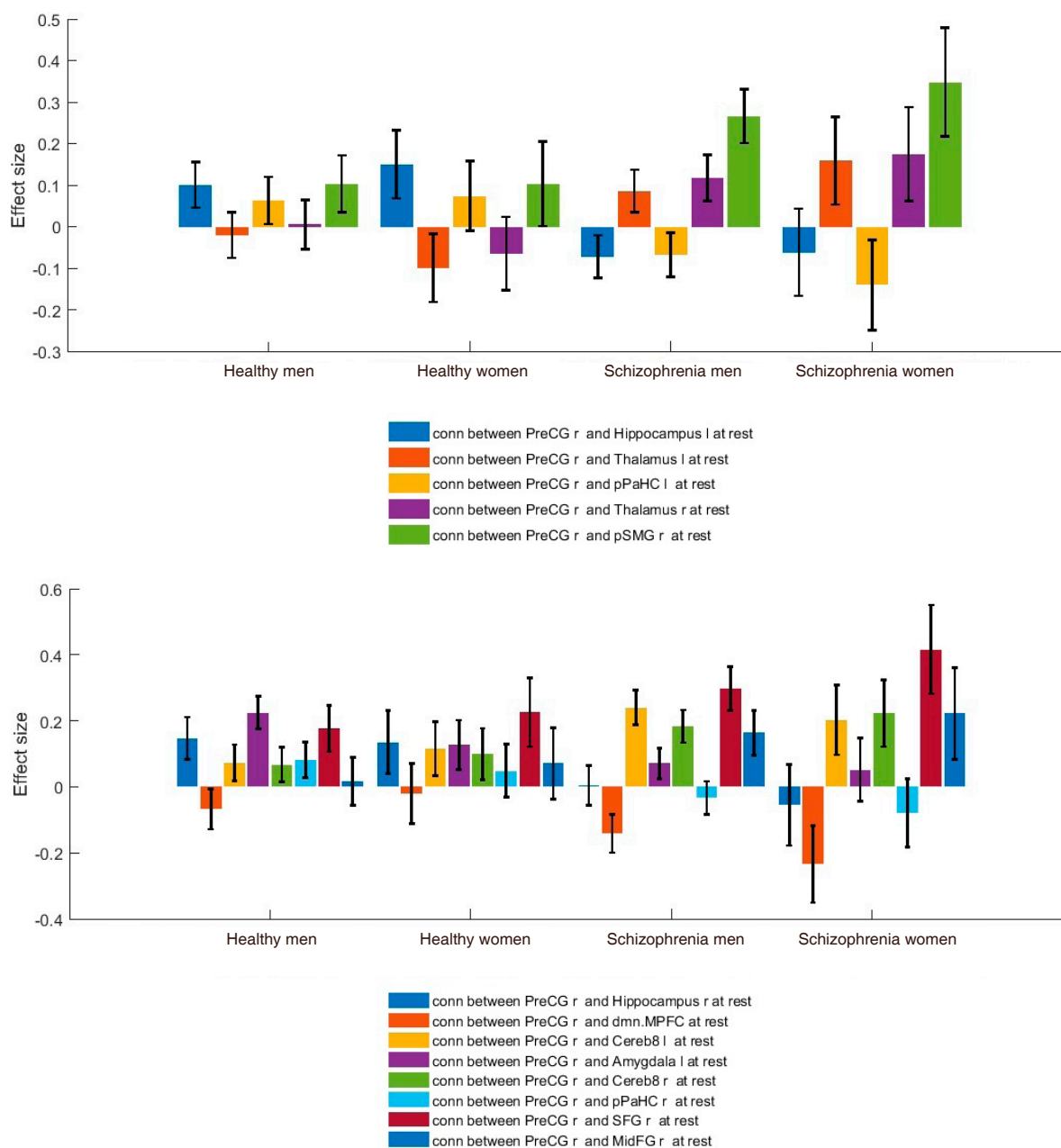
same in the male and female patients with schizophrenia. The resting-state functional magnetic resonance imaging data of healthy control subjects and patients with schizophrenia from the Centers of Biomedical Research Excellence dataset are used to examine the aberrant functional brain connectome in schizophrenia. Our results show precentral gyrus has abnormal communication with thalamus, hippocampus, pPaHC, pSMG and mPFC (pFDR = 0.05).





**Figure 3.** Functional connectivity difference between the left and right precentral gyrus between healthy controls vs. patients with schizophrenia (controls > schizophrenia). The PreCG connectivity in the healthy control, patients with schizophrenia, and their difference are shown in the first, second and the last column respectively and the two top rows are the superior views and the two-bottom row are the posterior views

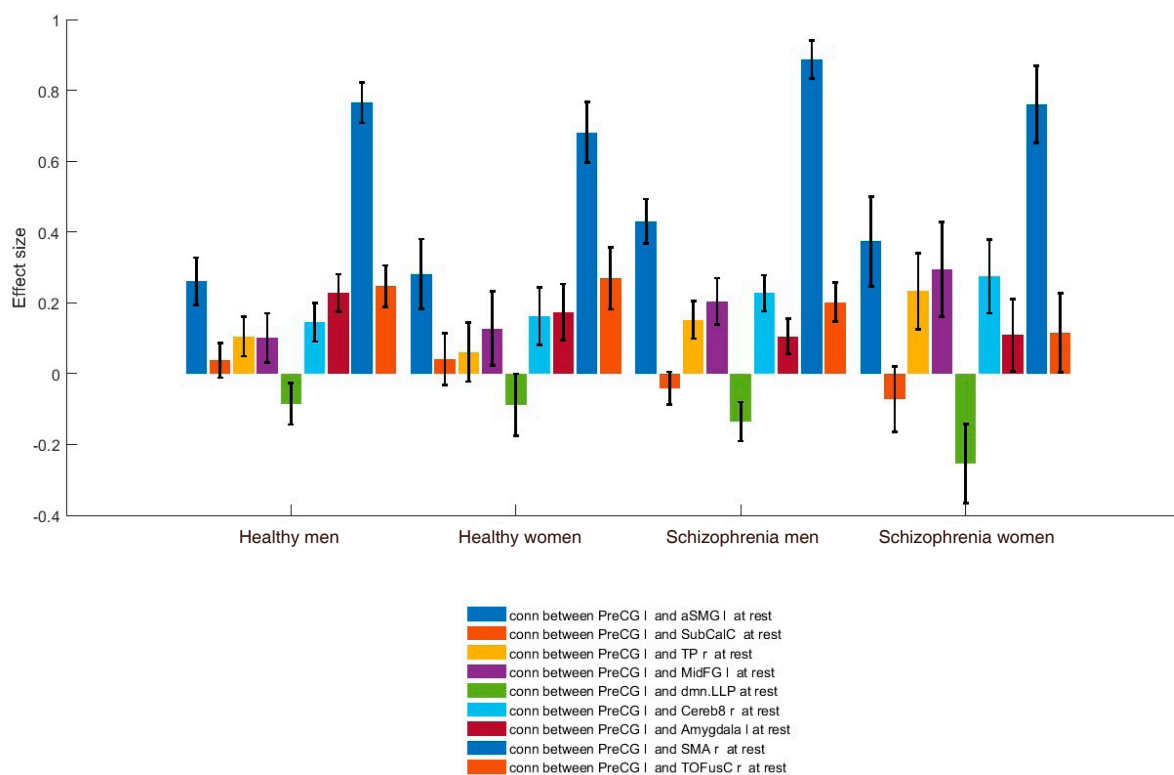
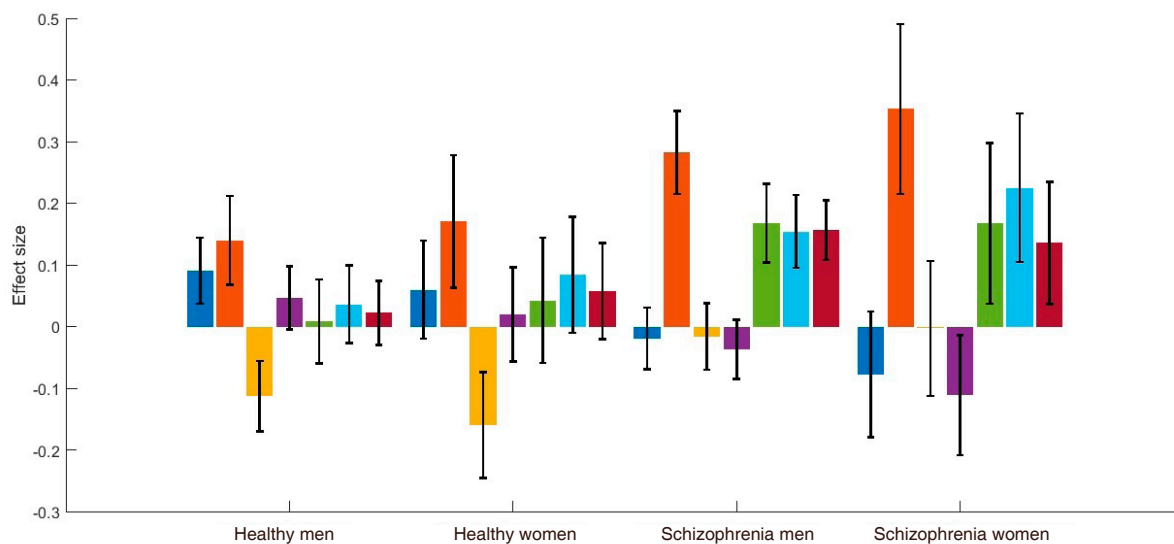
Here, we investigated abnormal connectivities of left and right precentral gyrus in male and female patients with schizophrenia. The region of interest based analysis carried out for the healthy control and patients with Schizophrenia data. To do this resting state functional magnetic resonance imaging data of healthy control subjects and patients with schizophrenia from Center for Biomedical Research Excellence are used

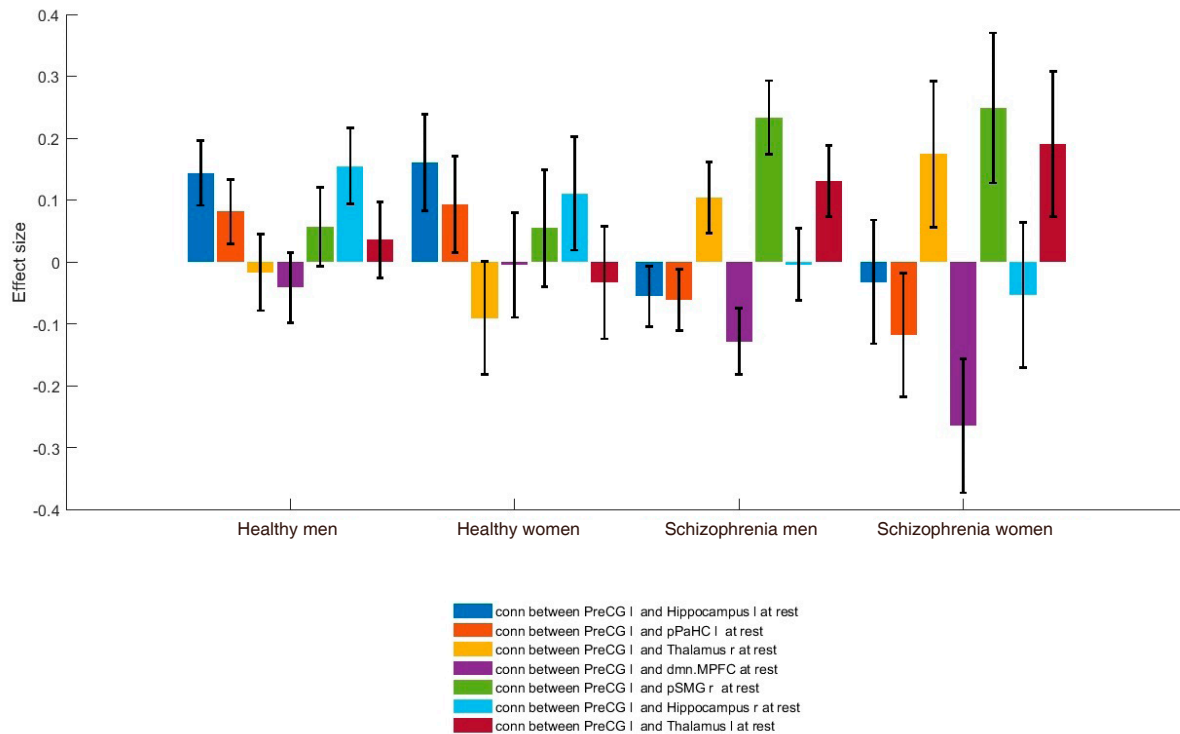


**Figure 4.** Right precentral gyrus functional connectivity difference between healthy controls and patients with schizophrenia (controls > schizophrenia)

to examine the aberrant functional brain connectome in schizophrenia which contains raw anatomical and functional MR data from 72 patients with schizophrenia and 75 healthy controls, ranging in age from 18 to 65 years old. We can summarize our results as follows: The precentral gyrus has abnormal communication with thalamus, hippocampus, pPaHC, pSMG and mPFC ( $P = 0.05$ ). The right precentral gyrus has unusual connectivities with Hippocampus l, pPaHC l Thalamus l, Thalamus r and pSMG r. The right precentral gyrus has unbalanced connectivities with Hippocampus l, pPaHC l, Thalamus r, MPFC, pSMG r, Hippocampus r and Thalamus l. The connectivity impairments of the PreCG in schizophrenia are different for male and female patients. This information is expected to provide a better understanding of altered functional connectivity of primary motor cortex in schizophrenia.







**Figure 5.** Left precentral gyrus functional connectivity difference between healthy controls and patients with schizophrenia (controls > schizophrenia)

## DECLARATIONS

### Authors' contributions

The author contributed solely to the paper.

### Data source and availability

Datas presented here are from the Center for Biomedical Research Excellence data set (COBRE). This data set is available at a previous study<sup>[7]</sup> and contains raw anatomical and functional MR data from patients with Schizophrenia and healthy controls, ranging in age from 18 to 65 years old. Resting fMRI, anatomical MRI, phenotype data for every participant including gender, age, handedness and diagnostic information are released.

### Financial support and sponsorship

None.

### Conflicts of interest

There are no conflicts of interest.

### Patient consent

Not applicable.

### Ethics approval

Not applicable.

### Copyright

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Case Report

Open Access



# Very rare upperdorsal intramedullary epidermoid with paraplegia: a case report

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## Abstract

Intramedullary upperdorsal epidermoid tumor is very rare. As far as we know, a small number of epidermoid tumors in the spinal cord have been reported in the journal for a long time. Most of the time, the spinal cord epidermoids are intradural extramedullary. We are reporting a case of a 21-year-old young man with paraplegia and upper dorsal pain for 6 months with normal physical findings. Magnetic resonance imaging scan shows that spinal intramedullary space occupying lesions in the dorsal 2/3 (D2/3) level. Total clearance was accomplished by performing laminectomy. Day to day paraplegia was improved. Histopathology was confirmed as an epidermoid tumor from two different centers.

**Keywords:** Upperdorsal, epidermoid, intramedullary

## INTRODUCTION

Epidermoids are rare before late childhood and have slight female predominance. Cervical and upper thoracic epidermoids are rare and conus is the most common site. Epidermoid tumors are usually located intradural extra medullary, but conus/cauda equina may have intramedullary component (completely intramedullary lesions are rare)<sup>[1]</sup>. An epidermoid cyst is a slow growing indolent rare lesion<sup>[2]</sup>. This tumor involving the spinal cord is quite uncommon. The opinion of the majority of authors is that these tumors arise from displaced normally developing somatic cells<sup>[3]</sup>. The vast majority of intraspinal epidermoid tumors are intradural and extramedullary. They are commonly associated with a dermal sinus and occur usually in the lumbosacral segments. In 1962, Manno *et al.*<sup>[4]</sup> in a review of all reported cases, found only



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5 tumors that they classified as being intramedullary. The incidence of intraspinal epidermoid cysts in children is 3% and in adults is 1%<sup>[1]</sup>. True intramedullary epidermoid cysts are rare, with < 60 cases having been reported in the literature since the 1st reporting of the entity by Chiari in 1833. Of these, a very few have detailed radiographic evaluation. Intramedullary epidermoid cysts are common in the dorsal and lumbosacral region. Regions with two frequent localizations are T4-T6 and T11-T12, while only three cases have been reported with cervical cord involvement<sup>[5-7]</sup>. However, our case showed that there is epidermoid in the dorsal 2/3 level, which is very uncommon site and no association with dermal sinus.

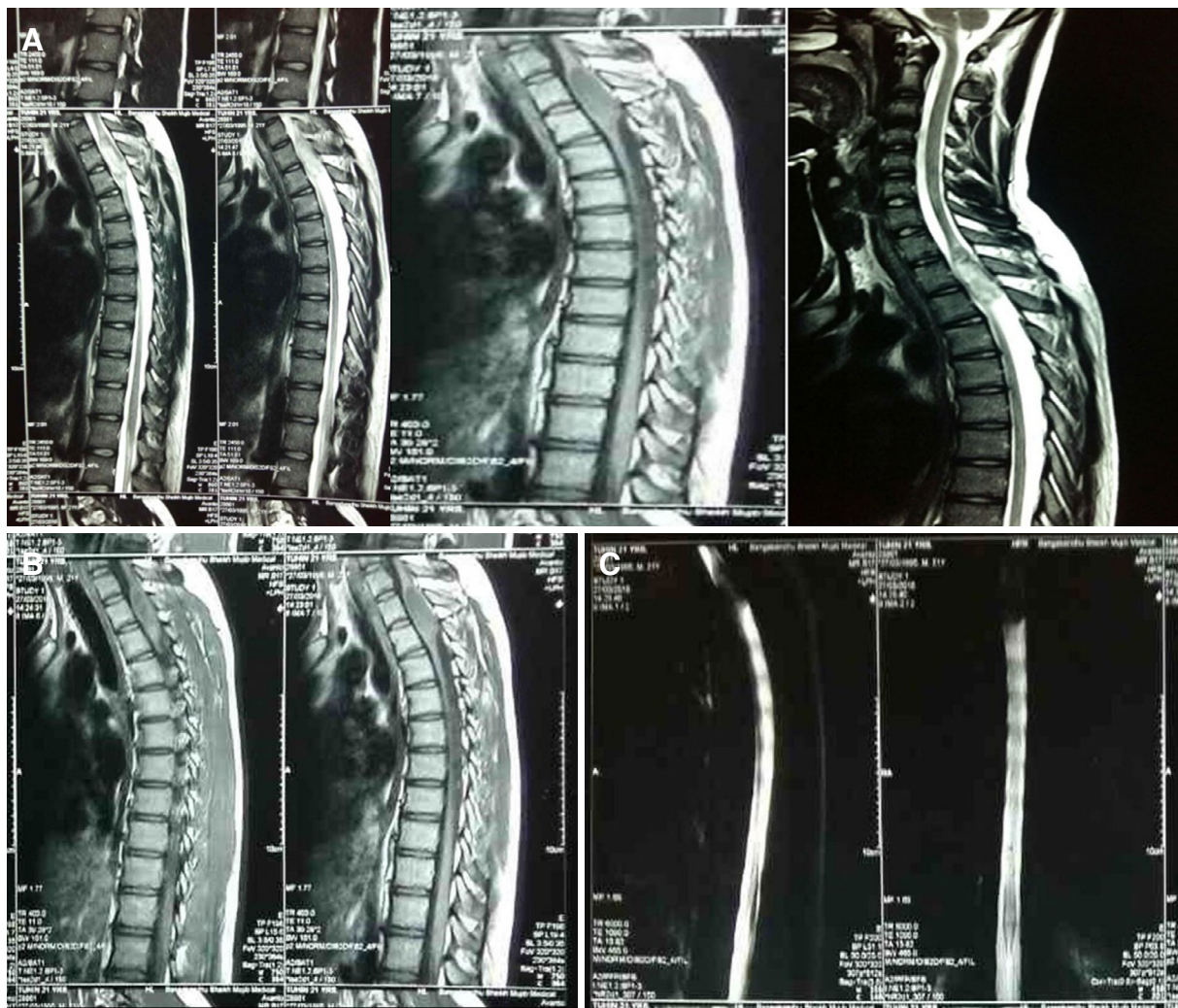
## CASE REPORT

A 21-year-old male was suffering from weakness of both lower limbs for 6 months, difficulty in walking for same duration, diminished sensation below mid chest for same duration along with constipation for 5 months and bladder disturbance for 1 month with erectile dysfunction. Weakness of both lower limbs which was insidious in onset, gradually progressive in nature, more on left side in comparison to right side of body below mid chest. His weakness was so severe nowadays that he encountered difficulties in walking and it was difficult to lift his body from kneeling position. Sometimes shoes run out of his feet. He complained about the numbness in his mid chest and diminished sensation of all modalities below mid chest and also complained of occasional incontinence of urine for last 1 month. He also complained of pain in back of mid chest for 3 months which was dull aching, mild in severity. There was no history of rest pain or night pain, and no definite aggravating or relieving factor. He didn't give any history of trauma to back or any part of body, and there was no scar mark over back fever, cough or hemoptysis. Clinical examination showed upper limb normal and muscle power of lower limbs Medical Research Council grade 3, all jerks exaggerated and planter extensor with ankle and patellar clonus were present. Sensory level dorsal 4 and coordinations and gate could not be assessed. Regarding spine examinations revealed normal. Magnetic resonance imaging (MRI) of dorsal spine showed intramedullary cyst like space occupying lesions at the level of D2/3 which was contrast uptake only margin of the lesions [Figure 1]. MR myelogram revealed that it completely cut off the sign of cerebrospinal fluid (CSF) flow [Figure 1]. Operation is performed by laminectomy and complete removal of tumor. Intraoperative tumor exhibited a typical pearly white appearance, such as other epidermis-like tissues, which were removed by piece meal approach. Only the capsule that is firmly attached to the capsule can be removed. After proper homeostasis dura closed and skin also closed in layers. Postoperatively, muscle power was decrease and subsequent follow-up gradually improved along with autonomic functions. Now he can walk without support but better with aid. Confirmation of histopathology as epidermoid carcinoma from two deferent centers [Figure 2].

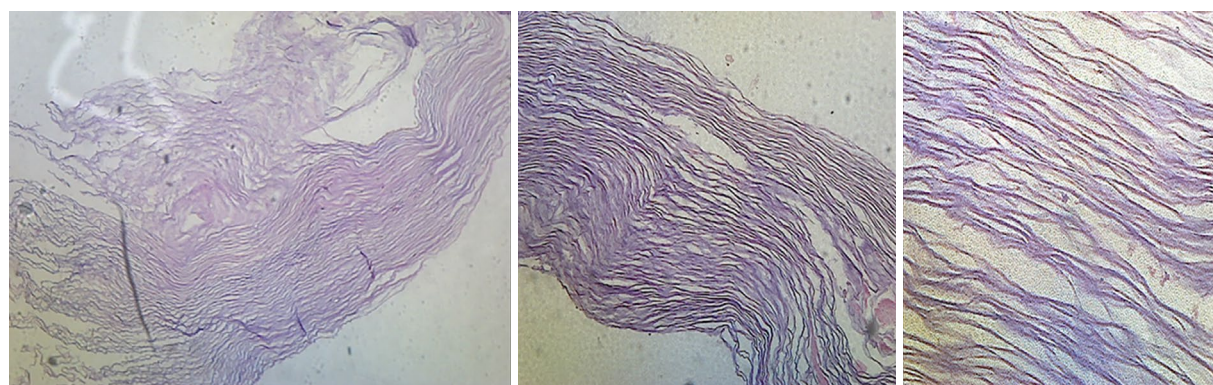
## DISCUSSION

Epidermoid cysts (sebaceous cysts) are benign congenital lesions of ectodermal origin. Intracerebral epidermoid cysts are rare and may account for approximately 1.5% of all intracranial epidermoids and approximately 1% of all intra-cranial tumors. Epidermoids usually present around 20 to 40 years of age and occur in both men and women with the same frequency. They can be congenital or acquired. They can be both intradural and extradural<sup>[8]</sup>. Epidermoids are in good condition, smooth, lobulated, cystic lesions. Histologically, their inner layer is composed of stratified squamous epithelium with a layer of capsule. They tend to slowly enlarge as epithelial cells desquamate, forming keratin and cholesterol crystals in the center of the lesion. Handa *et al.*<sup>[9]</sup> analyzed the aspirates of epidermal inclusion body of the epidermis to determine the cytological characteristics. Epidermoid cysts often occur in older age groups and show slower natural progression than most craniopharyngiomas. The epidermoid cyst wall consists of multiple layers of keratinized squamous epithelium which is located in the outer layer of collagen. The contents are more likely to be solid, keratinous, rather than the typical craniopharyngiomas oily fluid. The center of larger cysts sometimes degenerates, the keratin flakes are replaced with greasy brownish fluid that contain cholesterol crystals<sup>[10]</sup>. However, the reported case was a 21-year-old boy, which may be a congenital cause of no history of trauma or previous surgery. Pathogenesis of spinal epidermoid is either congenital or acquired.





**Figure 1.** MRI of dorsal spine T1, T2, contrast films and MR myelogram. (A) MRI of dorsal spine T1 and T2 weighted images shows space occupying lesions at D2/3; (B) MRI of dorsal spine with contrast shows contrast uptake only periphery of the lesions; (C) MR myelogram shows gross obstruction of CSF flow. MRI: magnetic resonance imaging; CSF: cerebrospinal fluid



**Figure 2.** Photomicrograph of intramedullary epidermoid showing lamellated keratin (hematoxylin and eosin stain: ×4, ×10, ×40)

Due to the shift of ectodermal contents during neural tube closure, most of spinal epidermoid are congenital. Congenital epidermoid cyst can be associated with other abnormalities, e.g. hemivertebra, dermal sinus, spina bifida, syringomyelia, but they can also occur independently. It is reported that about



10% of the overlying bone defect is possible, but less frequent than in dermoid or some extramedullary epidermoids<sup>[2,4,11,12]</sup>. Dermoid and epidermoid tumors are usually intradural extramedullary (60%) or intramedullary (40%). The lower thoracic and lumbar regions are the most common locations. Conventional radiographs are generally normal but may demonstrate benign spinal canal widening with flattening of the pedicles and laminae. On computed tomography these tumors are usually seen as well demarcated masses are similar to the attenuation of CSF. The presence of calcification is more suggestive of a dermoid than an epidermoid tumor. Again, there may be focal osseous erosion or spinal canal widening. On MRI, dermoids are typically hypointense to hyperintense on T1 with variable signal intensities that reflect fat (hyperintense on T1) or calcium (decreased signal intensity on T1). Epidermoids on T1 are usually equal signal. Both tumors showed increased signal intensity on T2-weighted images. Typically, these tumors do not enhance after contrast administration and may demonstrate restricted diffusion<sup>[13]</sup>. In our case, MRI showed mixed intensity on T2 film and isointense on T1 film with contrast enhancement within the margin of the lesions [Figure 1]. Before operation it was confused regarding actual diagnosis of this lesion but per operative appearance and histopathology was confirmed the diagnosis of upper dorsal (D2/3) intramedullary epidermoid.

In conclusion, upper dorsal inamedullary epidermoids are simply rare lesions in neurosurgical practice. Only a few literatures showed spinal intramedullary epidermoid in different areas rather than upper dorsal. We report this case as a rare entity and found intramedullary epidermoid at the abnormal locations.

## DECLARATIONS

### Authors' contributions

Conception, diagnosis, design, and manuscript editing: Rahman MA

Manuscript preparation: Hossain MA

Histological diagnosis: Habib S

Technical and manuscript revision: Barua KK

Literature search: Chaurasia BK

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There are no conflicts of interest.

### Patient consent

It was obtained from the patient.

### Ethics approval

Not applicable.

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Commentary

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## Sleep disturbance and treatment adherence: commentary on the study by Bosch *et al.* (2016)

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Treatment adherence is essential for optimizing healthcare outcomes, especially in individuals with schizophrenia or depression<sup>[1,2]</sup>. Previous studies identified several factors that predict treatment adherence, including psychiatric diagnoses, patients' characteristics, side-effects of psychotropic agents, symptom relief, and the patient-doctor relationship<sup>[3-5]</sup>. However, specific predisposing factors for treatment nonadherence in patients with psychiatric disorders is unknown. The present study by Bosch *et al.*<sup>[6]</sup> focused on the quality of sleep as one of the factors that could help to explain treatment nonadherence in patients with schizophrenia or depression. Their findings show that in patients with schizophrenia, more severe negative symptoms and better quality of sleep was associated with better treatment adherence. By contrast, in patients with depression, symptom severity or quality of sleep was not associated with treatment adherence.

The present study is noteworthy for its uncomplicated design with the use of well-established and standardized rating scales, making it easily replicated anywhere throughout the world. In addition, the merit of the current study findings can also be easily applied to actual clinical settings because all participants are outpatients of a clinic, at which most patients with schizophrenia and depression have been treated.

However, as Bosch *et al.*<sup>[6]</sup> noted the study's findings must be interpreted carefully. For example, treatment adherence was measured by self-reported sleep logs. Although self-reporting is a convenient survey method, it can produce unreliable responses that are influenced by such factors as psychiatric symptoms, cognitive function, premorbid characteristics and pharmacological intervention, which may preclude



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accurate measurement of “real” treatment adherence. In fact, it is unclear whether sleep disturbance, a major comorbid condition of schizophrenia and depression, directly affects treatment adherence. According to previous reviews of patients with schizophrenia, sleep disturbances are not predisposing factors for medication nonadherence<sup>[7,8]</sup>. Therefore, the study’s findings might only imply that schizophrenic patients with severe negative symptoms or better quality of sleep tend to complete the sleep log form and to submit it to their physicians.

In spite of the challenges in acquiring valid measurements of treatment adherence, the study illustrates key steps that are needed for the evidence-based intervention. It gives us a better understanding of the possible difference of the reasons related to nonadherence between schizophrenia and depression observed in this study, which may elicit a clue to elucidate the possible differences of underlying mechanisms of sleep disturbance among psychiatric disorders. Studies examining factors to maintain the treatment adherence has always been extensively conducted as one of the important topics to prevent the relapse of various psychiatric disorders since effective psychopharmacological treatments were established in the 1950s. However, the relative importance of these factors contributing to treatment adherence varies depending on the psychiatric disorders. Thus, further research is needed to identify specific risk factors in treatment nonadherence for each psychiatric disorder.

## DECLARATIONS

### Authors’ contributions

Drafted the manuscript text, developed the intellectual ideas, made the suggested revisions, and approved the final version to be published: Ishizuka K, Inada T

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Commentary

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# Customized autophagy: a long way to go

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The survival of any eukaryotic cell may have a unified theory i.e., energy production and clearance of unwanted organelles or pathogens which may be biological or non-biological. Survival of species over time depends not only on survival of cell but also on its ability to replicate and produce progeny. For these functions, intricate genetic, immunological responses including innate and adaptive immunity and congenial environment are needed. Autophagy is one of such mechanism and considered as a housekeeping system of a eukaryotic cell. Takeshige *et al.*<sup>[1]</sup> in 1990's first time elucidated the underlying mechanism for "autophagy" in yeast and showed that same type of fundamental mechanism is used by cells for degrading and recycling cellular components for which the group leader Yoshinori Ohsumi has been awarded Nobel prize in 2016. In last three decades, the role of autophagy has been extensively studied to understand the pathophysiology and to derive possible treatment options in both acute and chronic neurological diseases such as stroke, trauma, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis *etc.*<sup>[2-4]</sup>. Recently, the role of autophagy has been evaluated in neuroinfectious diseases especially to understand reactivation of latent virus<sup>[5]</sup>, persistence and replication of RNA virus<sup>[6]</sup>, immune enhancement leading to severe disease manifestations and survival of pathogenic organism against a hostile antibiotic treatment evading its action leading to drug resistance<sup>[7]</sup>. This review article by Sahu and Ter<sup>[8]</sup> has reviewed the role of autophagy in central nervous system (CNS) infection.

Amongst the different types of autophagy, macroautophagy is the most extensively studied and well characterized<sup>[9,10]</sup>. The role of micro-autophagy, chaperon mediated autophagy and xenophagy in central nervous system infections yet to be evaluated for bed side application. The immune regulation in CNS is quite different from systemic immune regulation. CNS mostly depends on microglial mediated immune regulation in presence of normal blood brain barrier and blood-cerebrospinal fluid barrier. However, CNS may suffer from double crash immune dysregulation in presence of CNS infection due to haematogenous



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dissemination of virus, bacteria, parasite or fungus, or meningitis in which natural barriers are lost<sup>[11]</sup>. Autophagy activation in this situation may be due to adaptive immune signalling through pattern recognition or by secretive pro-inflammatory cytokines (for example tumor necrosis factor- $\alpha$  and interferon- $\gamma$ ) following infections<sup>[12]</sup>. Autophagy can go in both ways, its activation may clear the micro-organism or the micro-organism may use autophagic activation for their benefit and survival<sup>[12]</sup>. This immune mediated autophagic process is highly regulated by a number of up and down regulating genes<sup>[13]</sup>. This may be the reason why some organisms provide different disease severity in different individuals or even in the same individual in the subsequent infection. There are many unresolved questions - Does the different organ system have customized autophagy operating system or have uniform operating system? How much autophagy activation is needed for clearance of pathogens and development of protective adaptive immunity? Is it possible to explore the survival autophagy response in adverse situation, the way saprophytic bacteria lives days to years? The resolution of these questions may pave the way for potential new treatment.

## DECLARATIONS

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Concept, literature search, manuscript preparation and review: Kalita J

Concept, manuscript review: Misra UK

Literature search, manuscript review: Kumar A

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Commentary

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# The emerging TDP-43 proteinopathy

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Currently, neurodegenerative diseases are viewed as proteinopathies. In this context, a specific protein or peptide is involved in the pathogenesis of the disease by missfolding, polymerization, reduced degradation and final accumulation in the form of insoluble inclusions leading to neurodegeneration by various interacting mechanisms<sup>[1,2]</sup>. In Alzheimer's disease extracellular beta amyloid peptides and intracellular hyperphosphorylated tau proteins accumulate in the brain. In parkinsonian syndromes alpha synuclein ( $\alpha$ -Syn) or 4R tau isoforms are found in various cytoplasmic inclusions.

In about 50% of patients with frontotemporal lobar degeneration (FTLD)<sup>[3]</sup> and about 90% of patients with amyotrophic lateral sclerosis (ALS)<sup>[4]</sup>, the responsible protein is the transactive response DNA binding protein-43 (TDP-43), forming ubiquitinated inclusions. The two clinical conditions may coexist in the same patient or the same family with TDP-43 being the major culprit in the ALS-FTLD spectrum<sup>[5]</sup>.

In an elegant study, Dong and Chen<sup>[6]</sup> extensively reviewed the pathogenetic mechanisms of ubiquitinated TDP-43 in ALS, including abnormal aggregation with pathologic accumulation (oligo- and finally polymerization), redistribution from the nucleus to the cytoplasm, reduced clearance by autophagy and/or the ubiquitin-proteasome system, neurotoxicity and/or loss of function, complex interactions with other proteins and RNA affecting their function and prion-like behavior and spreading. Such an understanding of TDP-43 and its role in the mechanisms of the resulting proteinopathy, may prove to be important in two aspects, diagnosis and treatment.



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## DIAGNOSIS

The various peptides and proteins involved in neurodegenerative diseases are secreted/released in the extracellular space and finally in the cerebrospinal fluid (CSF) by various mechanisms and some of them can be measured. Tau protein, either total or phosphorylated (phospho-tau) and beta amyloid peptide with 42 or 40 amino acids ( $A\beta_{42}$ ,  $A\beta_{40}$ ) can be reliably measured in the CSF, and currently, are recognized as helpful in the diagnosis of Alzheimer's disease (inclusion)<sup>[7]</sup> or its discrimination from other types of dementia, including the FTLDs (exclusion of AD)<sup>[8,9]</sup>. Thus, the CSF biochemical profile of AD is well recognized (increased tau and phospho-tau, decreased  $A\beta_{42}$ ). In synucleinopathies,  $\alpha$ -Syn is an emerging biomarker for Parkinson's disease, Dementia with Lewy Bodies and Multiple System Atrophy. A lot of studies have been conducted, while others are on their way towards better characterization of  $\alpha$ -Syn isoforms, antibodies used to recognize different epitopes and elimination of confounding factors interfering with preanalytical and analytical aspects, all with the purpose of establishing  $\alpha$ -Syn as a reliable biomarker in these disorders<sup>[10]</sup>.

On the other hand, the biochemical profile of TDP-43 proteinopathies has only recently begun to be explored and data are scarce and conflicting. Recently, however, CSF TDP-43 (either alone or in combination with other markers such as tau) has emerged as a candidate biomarker of ALS and FTLD and its levels are increased in both conditions<sup>[11,12]</sup>. Extensive investigation in order to determine and optimize its diagnostic potential is currently under way. Given the heterogeneity of proteinopathies, especially in FTLD (TDP-43 proteinopathies vs. tauopathies and others) and even within the TDP-43 proteinopathy in both ALS and FTLD (types A, B, C and D), correcting CSF diagnosis may, additionally, prove helpful in the correct classification of patients in clinical trials<sup>[13]</sup>.

## TREATMENT

Current treatments approved for ALS have modest effects and for FTLD are only symptomatic. The lack of success of pharmacological trials in such patient populations may be due to clinical trials being performed either too late in the course of the disease, with too short duration, inclusion of heterogeneous groups of patients, or insufficient knowledge about the specific clinical features and/or pathogenesis. The various steps in TDP-43 distribution, cytoplasmic accumulation, phosphorylation, ubiquitination, aggregation, autophagy, degradation, exosome secretion and toxicity may all be hypothetical targets of therapeutic interventions<sup>[13]</sup> and some of them are currently tested *in vitro* and in animal models, including improved degradation, reduced neurotoxicity and antibodies against TDP-43<sup>[6]</sup>.

In conclusion, mechanisms of TDP-43 abnormal accumulation and toxicity, extensively reviewed by Dong and Chen<sup>[6]</sup> in addition to measurement of CSF TDP-43 levels as a diagnostic tool, are currently hot topics relevant to the majority of ALS and roughly half of FTLD patients.

## DECLARATIONS

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Literature search: Paraskervas GP, Bourbouli M

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### Conflicts of interest

There are no conflicts of interest.

## Patient consent

Not applicable.

## Ethics approval

Not applicable.

## Copyright

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Editorial

Open Access



# Bone mineral density in patients with multiple sclerosis

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Patients with multiple sclerosis (MS) have higher incidence of low bone mineral density (BMD) compared to normal subjects<sup>[1]</sup>. In their recent paper, Olsson *et al.*<sup>[2]</sup> studied trabecular bone score (TBS) employing dual-energy X-ray absorptiometry (DXA) in lumbar spine (L2-L4), providing measurement of the bone microarchitecture. Their study was performed in 260 MS patients (202 being females) and compared with the results of 6310 healthy individuals taken from the TBS software database. Patients' mean age was 43 ± 10 years (20-71) and control's group age was from 45 to 85 years old. The authors found that trabecular bone score was not altered in MS from the control population. They concluded that although BMD may be affected in MS patients, the bone microarchitecture seems to remain intact. However, an association was found with lower TBS and higher age, gender, expanded disability status scale (EDSS) ≥ 6, disease duration, smoking and menopause.

The TBS is an assessment of bone quality correlated with bone microarchitecture and consists of an indicator for the risk of osteoporosis. It is actually a reflection of the structural condition of the bone microarchitecture.

This study had some limitations as reported by the authors. Due to the type of the study (retrospective/cross-sectional), causality could not be determined and there was lack of medical information such as bone fractures, menarche age and lactation. Finally, the control group was not selected by the authors but was taken by the TBS software, where data is provided for individuals over 45 years old up to 85 years old that is problematic in comparison to a MS young to middle age peak demographic.



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Several risk factors for low BMD in MS patients have been reported such as, disease duration, total steroid dose, EDSS score ( $> 3$ ), immobility and vitamin D deficiency<sup>[3-7]</sup>. Vitamin D deficiency, especially during relapses, suggests that it could regulate clinical disease activity and it may be a modifiable MS risk factor. Vitamin D action is mediated through its specific receptor (VDR). Various polymorphisms of the VDR may affect vitamin D function and may be linked to osteoporosis and MS<sup>[8,9]</sup>.

Male MS patients exhibit reduced bone mass disproportionately to their age and ambulation<sup>[10]</sup>. Furthermore, reduced mobility and chronic low-dose glucocorticoid treatment in male MS patients is linked to increased osteoporosis and muscle wasting<sup>[11]</sup>. In premenopausal female patients, the length of the disease and their relative immobility were linked to low BMD<sup>[12]</sup>. In addition, menarche age  $\geq 13$  years and breast feeding may be associated with reduced BMD<sup>[12]</sup>. Another predisposing factor leading to reduced BMD in MS females includes 25-OH-Vit D3 deficiency and secondary parathyroid hormone (PTH) elevation<sup>[13]</sup>.

Overall, the study by Olsson *et al.*<sup>[2]</sup> provides novel questions for the existence of bone loss in MS patients and the participation of bone microarchitecture in this process. It is clear that further studies are needed to elucidate the role of bone microarchitecture during bone loss, as evaluated with the TBS. Due to the limitation of the study<sup>[2]</sup>, several information could not be provided such as the association of TBS with the fertility history or gynecological factors. Considering the physiological bone loss after a certain age and the fact that 80% of the studying patients were females, it would have been interesting to see the role of TBS according to gynecological factors in pre- and post-menopausal status.

In conclusion, the etiology of BMD loss in MS patients is yet to be answered and future studies of larger number of patients would help further elucidate the etiology of bone loss and perhaps the participation of bone microarchitecture during this process.

## DECLARATIONS

### Authors' contributions

Conceived and wrote the manuscript: Sioka C

Reviewed and corrected the manuscript: Fotopoulos A

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Case Report

Open Access



# Demyelinating central nervous system lesions, following the use of tumor necrosis factor alpha antagonist

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## ABSTRACT

Tumor necrosis factor alpha (TNF $\alpha$ ) antagonists have been a valuable tool in treating patients with rheumatoid arthritis (RA), but reports and case series of neurological adverse events due to TNF $\alpha$  antagonists have been reported. Furthermore, central nervous system (CNS) lymphoma always remains a remote, yet a worrisome complication in RA patients, especially in those under treatment with methotrexate (MTX). We present a female patient with RA with tumor-like active demyelinating lesions attributed to TNF $\alpha$  antagonist, confirmed by an FNA biopsy, otherwise clinically and radiologically challenging to distinguish from CNS lymphoma. A 72-year-old female patient with RA under TNF $\alpha$  antagonist and MTX treatment was presented with neurological symptoms and signs. The brain MRI revealed four tumor-like contrast-enhancing lesions bilaterally, the demyelinating nature of which was delineated by the FNA biopsy. A full clinical and radiological recovery was achieved after the TNF $\alpha$  antagonist was permanently withdrawn. Patients with RA under anti-TNF agents and MTX are predisposed to complications such as CNS lymphoma and CNS demyelination. This case uniquely highlights the physicians' vigilance in pursuing these complications and the usage of the FNA when tumor-like appearances on the brain MRI convolute the final diagnosis.

**Keywords:** Anti-tumor necrosis factor complications, demyelination, rheumatoid arthritis



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## INTRODUCTION

Tumor necrosis factor alpha (TNF $\alpha$ ) antagonists represent a revolutionary therapeutic choice for many inflammatory diseases such as rheumatoid arthritis (RA)<sup>[1,2]</sup>. Although they are generally considered as a low-risk intervention, critical side effects of their use may develop that require awareness and prompt action, such as instant discontinuation of TNF $\alpha$  antagonists. Furthermore, RA patients have a high risk of a site-specific lymphoma regardless of the lymphoma type<sup>[3]</sup>.

We present a case of RA treated with TNF $\alpha$  antagonists and methotrexate (MTX), with tumor-like active-demyelinating brain lesions effectively confirmed by an FNA biopsy, as it is both clinically and radiologically challenging to differentiate from central nervous system (CNS) lymphoma.

## CASE REPORT

A 72-year-old woman, with a diagnosis of rheumatoid arthritis for the past 18 years, was admitted to the Department of Neurology at the University Hospital of Ioannina with symptoms of dizziness, headache and walking instability, that gradually developed in the last week before her admission. The patient has been receiving treatment with TNF $\alpha$  antagonist (etanercept) and MTX for the last 18 months.

The patient underwent a complete physical examination and a detailed neurological evaluation which included both lumbar puncture and brain magnetic resonance imaging (MRI). The patient had mild left hemiparesis and ataxia. The lumbar puncture showed a normal cerebrospinal fluid (CSF) cell count, a mild elevation in CSF protein levels (57 mg/dL) and an elevated CSF IgG index (0.857). CSF was negative for malignant cells.

The brain MRI showed bilateral contrast-enhancing lesions in the cerebral hemispheres with moderate perilesional edema and enhancement [Figure 1]. Because malignant lymphoma was considered first in the differential diagnosis, no corticosteroids were administered and the patient was promptly referred for an FNA biopsy. The histological examination, which included luxol fast blue staining, showed findings compatible with lesions of demyelinating nature. The anti-TNF agent was considered as a potential causative factor for the demyelination, thus was withdrawn from the patient. No other treatment was initiated.

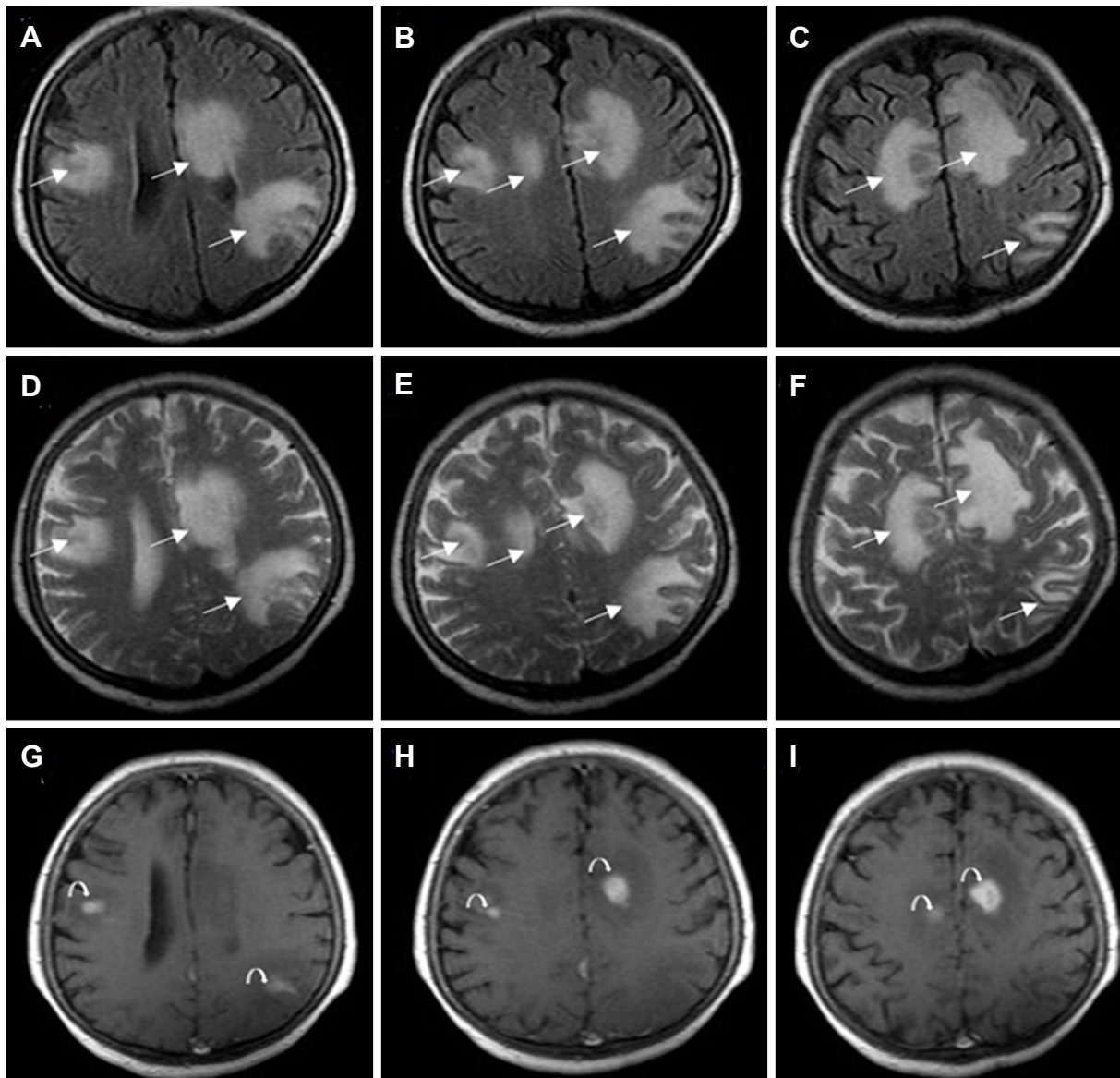
The patient, who has eventually been placed on interferon, was followed up every 2 to 3 months with appropriate laboratory monitoring, as well as with complete physical and neurological examinations. An MRI was repeated after a mean period of 6 months. Symptoms were completely resolved in 2 months with a dramatic retrieval of the imaging findings [Figure 2].

## DISCUSSION

This is the first case, to our knowledge, to show that an FNA biopsy has differentiated brain demyelination from CNS lymphoma in an RA patient, treated with TNF $\alpha$  antagonist and MTX. The TNF $\alpha$  antagonist was considered as the causative factor for the demyelinating lesions, which were resolved by withdrawing the medication.

Many adverse events, due to TNF $\alpha$  antagonists, have been published, including central nervous and peripheral nervous system demyelination, transverse myelitis, retrobulbar optic neuritis and more<sup>[4-6]</sup>. The mechanism of this demyelination is not perfectly outlined, since it is not certain whether anti-TNF $\alpha$  blockers unmask pre-existing demyelinating disorders or induce *de novo* demyelination of the CNS and peripheral nervous system<sup>[1,7]</sup>.

In a French national survey<sup>[5]</sup>, demyelination occurred in a median period of 10.2 months after treatment initiation, while etanercept was the reported agent in the majority of cases of CNS involvement<sup>[8]</sup>. Of interest



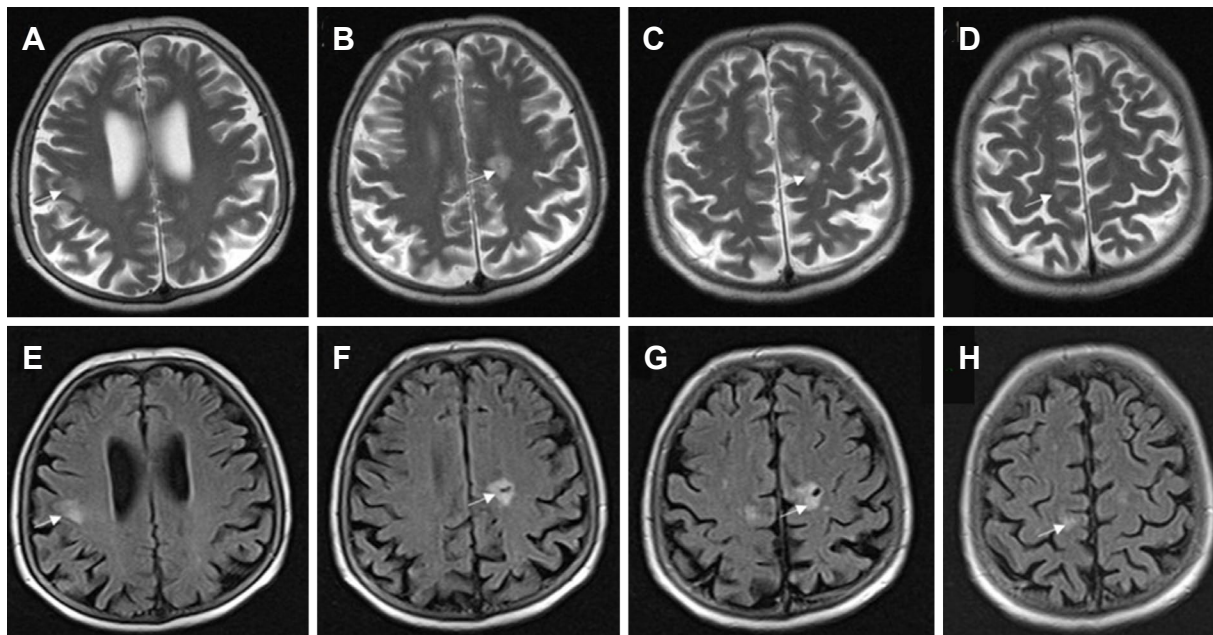
**Figure 1.** Axial fluid-attenuated inversion recovery (A-C) and T2-weighted (D-F) images show multiple large juxtacortical and periventricular hyperintense lesions (arrows). Axial post-contrast T1-weighted images (G-I) demonstrate that the lesions abnormally enhance, suggesting an active demyelinating process (curved arrows)

is the fact that, in most reported cases, the chronology of clinical events is suggestive of a causal relationship between anti-TNF $\alpha$  and induction of demyelination, but only few cases have shown a definite positive relationship, proven by rechallenge after re-admission of anti-TNF agents<sup>[5]</sup>. There is only one report in the literature of demyelinating lesions<sup>[9]</sup>, that is proven to be demyelinating after craniectomy and biopsy.

It is well known that patients with certain autoimmune and inflammatory disorders, such as RA, have an increased risk of developing malignant lymphoma<sup>[10]</sup> with an overall two-fold increase in lymphoma risk compared with the general population<sup>[3]</sup>. The possible mechanisms for this increased risk include the fact that RA results in persistent immunologic stimulation (which may lead to clonal selection and predispose CD5+ B cells to malignant transformation), and decreased number and function of T-suppressor lymphocytes<sup>[3]</sup>.

Furthermore, mature T/NK-cell lymphoproliferative diseases development is a frequent complication in RA patients treated with MTX<sup>[11]</sup>, which additionally may shorten the interval between the diagnosis of RA and





**Figure 2.** Follow up magnetic resonance imaging after 3 months. Axial T2-weighted (A-D) and fluid-attenuated inversion recovery (E-H) images reveal significant remission of the lesions (arrows)

lymphoma development when compared with that in MTX-naïve RA patients<sup>[12]</sup>.

Conclusively, in patients with RA treated with TNF $\alpha$  antagonists and MTX, both lymphoma and CNS demyelination may rarely occur. Patients with RA should benefit from a follow-up, including a brain MRI<sup>[7]</sup>. Since brain imaging findings of lymphoma and CNS demyelination often share similarities, especially in tumor-like active-demyelinating lesions, their distinction may prove to be challenging<sup>[9]</sup>. An FNA biopsy could be recommended in these cases, before proceeding any further with a craniotomy. Luxol fast blue staining (which is not routinely included in tumor histological protocols) should be requested to detect the potential demyelinating nature of the lesion<sup>[13]</sup>.

## DECLARATIONS

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### Authors' contributions

Data collection: Bogdos E

Data accuracy and integrity: Drosos A

Drafting: Bogdos E, Markoula S

Revising manuscript: Bogdos E, Markoula S, Zikou A, Voulgari P

Providing guidance and connecting all authors: Markoula S

Approval of final version of manuscript: Konitsiotis S

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### Conflicts of interest

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## Patient consent

Informed consent was obtained from the patient.

## Ethics approval

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Case Report

Open Access



# Hypoparathyroidism presenting with late onset seizures - a report of two cases from rural India

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## ABSTRACT

Hypoparathyroidism leading to hypocalcaemia is an important treatable cause of recurrent seizures. Neurological manifestations due to hypoparathyroidism include: seizures, paresthesia, depression, psychosis, extra pyramidal symptoms, and features of raised intracranial pressure. Seizures may be the presenting symptoms preceding other signs of hypocalcaemia. Primary hypoparathyroidism presenting for the first time as seizures in the elderly is quite rare. Here we report two cases of hypoparathyroidism presenting with seizures in the elderly as the sole manifestation of hypocalcaemia. The goal of this report is to seek attention to such an uncommon reversible treatable cause of seizures and to consider hypoparathyroidism in the work up of these patients.

**Keywords:** Late onset seizures, hypoparathyroidism, hypocalcemia

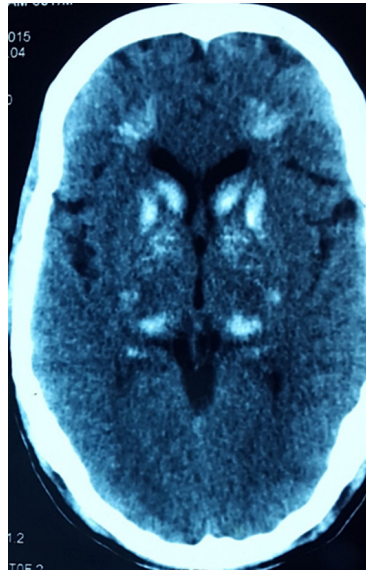
## INTRODUCTION

Hypoparathyroidism occurs when too little parathyroid hormone (PTH) is released from the parathyroid glands, or the released PTH does not work properly<sup>[1]</sup>. Common clinical manifestations of hypoparathyroidism include tingling and numbness, carpopedal spasm, neurocognitive dysfunction and seizures. Hypoparathyroidism and pseudohypoparathyroidism (due to deficient end-organ response to PTH) are the most familiar reasons for pathological basal ganglia calcification.



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**Figure 1.** NCCT head scan was done which showed bilateral basal ganglia calcification and many punctiform calcifications between cortical and subcortical parts

Invariably, hypoparathyroidism presents with a spectrum of clinical features as above, here we report two cases with late onset seizures being the only manifestation of primary hypoparathyroidism which is quite uncommon in literature.

## CASE REPORTS

### Case 1

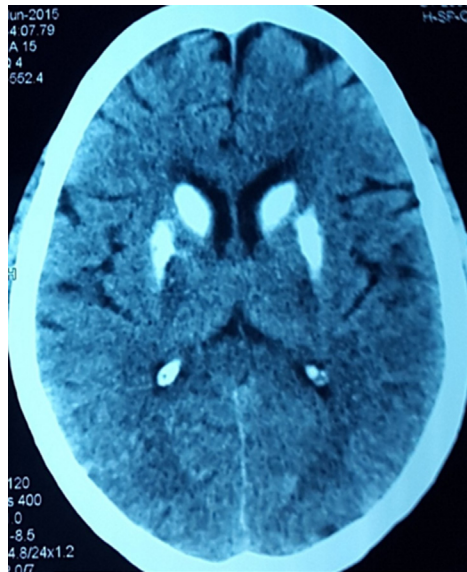
A 62-year-old right-handed male farmer presented to emergency department with 3 episodes of abnormal movement in the form of generalized muscles stiffness with eyes-rolling and associated with frothing, tongue bite and incontinence in a 2-h cycle. Each episode lasted for approximately 5 min and at the interval of 30-40 min. He had similar two episodes one year and one episode 15 days back. These were not preceded by any aura and each attack lasts for two to three minutes with postictal confusion for 15 to 20 min. He reported no history of neck surgery, muscle cramps, parasthesia, and psychiatric illness. He had cataract extraction surgery on the right eye 4 years back. Family and social history were unremarkable.

He was treated by some local practitioner with antiepileptic drugs (phenytoin 300 mg and clobazam 20 mg/day) without basic investigations. His vital signs, weight and height were normal with no dysmorphic features (no dry hairs or brittle nails). Neurological examination, including higher mental functions, cranial nerves, motor, sensory, reflexes and coordination examination were normal. There was no carpopedal spasm or any other signs of tetany like Chvostek's or Trousseau's sign. Computerized tomography scan head was done showing bilateral basal ganglia calcification and many punctiform calcifications between cortical and subcortical parts [Figure 1].

Electroencephalography (EEG) was normal. The laboratory investigations included complete blood count, erythrocyte sedimentation rate, routine chemistries, liver, and renal function test were normal. He was found to have a serum calcium level of 6.3 mg/dL (normal range 8.0-10.4 mg/dL) with a serum PTH level of 3.8 pg/mL (normal range 15-68 pg/mL), serum phosphorus 5.9 mg/dL (normal range 2.5-4.5 mg/dL) and magnesium 1.4 mg/dL (normal range 1.3-2.5 mg/dL).

### Case 2

A 65-year-old right-handed female housewife presented with history of episodic eye-rolling with generalized tonic-clonic movements of limbs and was associated with incontinence of urine and tongue bite, for



**Figure 2.** Computerized tomography scan of the head revealed bilateral basal ganglia

the duration of 6 months. She has 6-7 attacks/month and each attack last for 3-4 min without aura with postictal state for 30 min. There was no past or family history of epilepsy. She was on antiepileptic drugs carbamazepine 800 mg/day and clobazam 20 mg/day started by local practitioner. The patient did not have any symptoms of hypocalcemia. Her general examination was normal and there were no focal neurological signs.

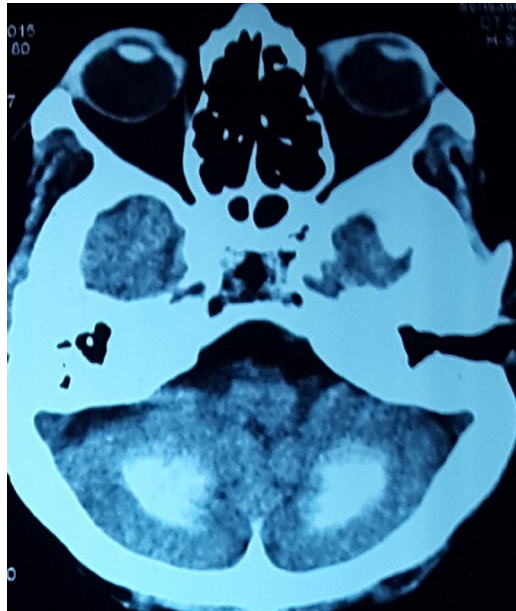
Signs of hypocalcaemia were elicited by inflating a cuff above systolic blood pressure over the arm for 2 min, provoking flexion of the wrist and metacarpophalangeal joints, hyperextension of the fingers, and flexion of the thumb (carpopedal spasm - Trousseau's sign), and by tapping the face just anterior to the ear, provoking twitching of the ipsilateral facial muscles (Chovstek's sign). Computerized tomography (CT) scan of the head revealed bilateral basal ganglia and cerebellar calcification [Figures 2 and 3]. She was investigated for hypoparathyroidism. Her serum calcium level was 5.9 mg/dL with a serum PTH level of 2.8 pg/mL and serum phosphorus 7.2 mg/dL. EEG showed runs of higher amplitude theta and delta activity with very marked response during hyperventilation.

Because of the presence of bilateral basal ganglia and cerebellar calcification on CT, the patients were investigated for hypoparathyroidism. Both patients had hypocalcemia, hyperphosphatemia with low serum PTH levels. The biochemical profile favored the diagnosis of primary hypoparathyroidism. Both patients were started on daily supplementation of 0.5 µg of calcitriol and 1000 mg of elemental calcium (calcium carbonate). The patients were discharged with oral calcium and vitamin D. The biochemical profile normalized, seizures were controlled, and eventually antiepileptic drugs were withdrawn. They did not experience any further seizures in the next 12 months.

## DISCUSSION

Hypoparathyroidism can be caused by congenital disorders (like Di George syndrome, mitochondrial cytopathies), receptor insensitivity (pseudohypoparathyroidism type Ia-c, II), surgery, autoimmune disorders (familial autoimmune polyglandular syndrome type I), or hemochromatosis, or can be idiopathic<sup>[2,3]</sup>.

Acquired chronic hypoparathyroidism is generally the after effect of unintentional surgical removal of all the parathyroid glands. Even rarer causes of acquired chronic hypoparathyroidism include radiation-induced damage and glandular damage in patients with hemochromatosis or hemosiderosis after repeated blood



**Figure 3.** Computerized tomography scan of cerebellar calcification

transfusions. Secondary hypoparathyroidism is a physiological state in which PTH levels are low in response to a primary process that causes hypercalcemia.

The essential capacity of PTH is to keep up the extracellular fluid (ECF) calcium concentration within a normal narrow range. The hormone acts directly on bone and kidney and indirectly on the intestine through its effects on synthesis of  $1,25(\text{OH})_2\text{D}_3$  to increase serum calcium concentrations; in turn, PTH generation is firmly directed by the concentration of serum ionized calcium. Any tendency toward hypocalcemia, is counteracted by an increased secretion of PTH. This in turn: (1) increases the rate of dissolution of bone mineral, thereby increasing the flow of calcium from bone into blood; (2) reduces the renal clearance of calcium, returning more of the calcium filtered at the glomerulus into ECF; and (3) increases the efficiency of calcium absorption in the intestine by stimulating the production of  $1,25(\text{OH})_2\text{D}_3$ .

Hypocalcemia produces hyperexcitability of nerve fibers with spontaneous and repetitive discharges. As a result, patients have perioral and distal numbness and parasthesiae, carpopedal spasm and diffuse muscle cramps. Latent tetany can be elicited by hyperventilation, by tapping the facial muscles (Chovstek's sign) or by occluding venous return from arm, resulting in carpopedal spasm (Trousseau's sign). In severe cases laryngeal muscle spasm and convulsions may develop. Respiratory arrest may also occur. Chronic hypoparathyroidism is associated with markedly abnormal skeletal microstructure, despite marked increases in bone mineral density.

Increased intracranial pressure, papilledema, and convulsions can also be present, and must be differentiated from severe tetany muscular spasms<sup>[4,5]</sup>. Rare extrapyramidal signs including parkinsonism and cerebellar signs have been reported. Mental changes include irritability, depression, and psychosis. The QT interval on the electrocardiogram is prolonged, in contrast to its shortening with hypercalcemia. Arrhythmias occur, and digitalis effectiveness may be reduced. Intestinal cramps and chronic malabsorption may also occur.

Seizures are a frequent complication: they have been reported in 20%-25% of patients with acute hypocalcemia and in 30%-70% of patients with idiopathic hypoparathyroidism<sup>[6]</sup>. Epileptic seizures may happen at any age and usually generalized tonic clonic with loss of consciousness. Non-convulsive status

has been also reported. Seizures may be the presenting symptoms preceding other signs of hypocalcemia such as chorea and tetany. Several types of partial motor seizures including jacksonian seizures may also be observed. If not treated the results of these convulsions may be very serious. Seizures are thought to occur due to hypocalcemia and intracranial calcification that occur in vascular and perivascular locations<sup>[7,8]</sup>.

Early EEG changes associated with hypocalcemia include evolution from alpha through theta and delta dominance. Runs of higher amplitude theta activity also appear and a very marked response to over breathing occurs. Other EEG findings (generalized spikes, sharp-waves burst of delta activity with sharp components). Generalized paroxysmal discharges and absence status epileptic have also been reported<sup>[9]</sup>. No correlation was found between calcium level and EEG changes and these changes typically revert to normal with correction of the serum calcium levels.

Eaton *et al.*<sup>[10]</sup> in 1939 first described basal ganglia calcification (BGC) in association with chronic hypoparathyroidism. Pathogenesis is obscure, but its occurrence with hypocalcemia signifies an important role of increased calcium-phosphorus complex formation. Radiological studies have found that calcification encompassing cerebral veins most habitually happen in the lentiform (putamen and globus pallidus) and the caudate cores of the basal ganglia; however, the factors that predispose individuals to basal ganglia calcification have not been identified<sup>[11]</sup>. Other areas affected by BCG include the thalamus, dentate nuclei, cerebral cortex, gray-white junctions, and the cerebellum<sup>[10,12]</sup>. Such intracranial calcification occurs in 0.3% to 1.5% of patients with hypoparathyroidism, and is often detected incidentally<sup>[13,14]</sup>. Treatment involves replacement with vitamin D or 1,25(OH)<sub>2</sub>D<sub>3</sub> (calcitriol) combined with a high oral calcium intake. For many patients, vitamin D in doses of 40,000-120,000 U/day (1-3 mg/day) combined with ≥ 1 g elemental calcium is satisfactory. Many physicians now use 0.5-1 µg of calcitriol in management of such patients, especially if they are difficult to control. It is critical to monitor therapy closely since overtreatment may result in hypercalciuria, hypercalcemia, renal stones, and nephrocalcinosis. Modi *et al.*<sup>[15]</sup> reported among 70 patients, seizures were present in 64.3% of patients with idiopathic hypoparathyroidism and they responded to antiepileptic drugs (AEDs) and calcium/1α(OH)D<sub>3</sub> during the follow-up and it was possible to withdraw AEDs in 71% of patients.

In both of our patients' basic investigations were not done before starting the antiepileptic drugs due to lack of facilities in this rural and remote part of country. Hypoparathyroidism is a rare yet treatable cause for seizures. For any patient having new onset seizures even in the elderly population basic investigations, including calcium profile, may uncover the hidden etiology.

The diagnosis of idiopathic hypoparathyroidism is often missed if presentation is in elder age group. The objective of this report is to increase awareness of such a rare, reversible cause of seizures and to consider hypoparathyroidism in the work up of these patients.

## DECLARATIONS

### Authors' contributions

Both the case workup and treatment: Verma A  
Manuscript writing: Kumar A

### Financial support and sponsorship

None.

### Conflicts of interest

There are no conflicts of interest.



## Patient consent

Consents from both patients were established prior to submission.

## Ethics approval

All treatment and study were performed in compliance with our institutional standard and the Declaration of Helsinki, No: 314 /UPUMS/Dean/2016-2017.

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Original Article

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# Surgical management of clinoidal meningiomas: 10 cases analysis

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## ABSTRACT

**Aim:** The purpose of this article is to advocate standard skull base technique for removing the clinoidal meningioma and to delineate the technique's advantages that aid in achieving an improved extent of tumor resection and enhancing the patients' overall outcome, specially their visual outcome.

**Methods:** A retrospective analysis was performed on 10 consecutive patients with clinoidal meningiomas who underwent surgical resection at the Bangabandhu Sheikh Mujib Medical University and other private clinics between May 2013 and July 2016. A standard pterional craniotomy technique consisting of extradural anterior clinoidectomy, coupled with optic canal unroofing and optic nerve sheath opening was used in all patients. All patients had thorough preoperative and postoperative ophthalmological evaluations. The follow-up period ranged from 6 to 42 months.

**Results:** Total resection was achieved in 5 (50.0%) of the 10 patients in this series. The majority of the patients with preoperative visual impairment experienced significant visual improvement 7 of 10 patients; 70.0%).

**Conclusion:** In the majority of patients with clinoidal meningiomas, total resection may be achieved with minimal complications. For large tumors encasing the optic nerve and internal carotid artery, or for those tumors causing preoperative visual impairment, use of the cranial base technique delineated in this study may lead to significant improvement in the patients' visual and overall outcomes.



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**Keywords:** Clinoidal meningiomas, pterional craniotomy, extradural anterior clinoidectomy

## INTRODUCTION

Anterior clinoidal meningiomas arise from the meningeal covering of the anterior clinoid process. In the classical neurosurgical literature, anterior clinoidal meningiomas have not been separated from medial sphenoid wing or inner sphenoid wing meningiomas<sup>[1,2]</sup>. However, accumulating anatomical knowledge and clinical experience has shown that anterior clinoidal meningiomas have unique anatomical and clinical characteristics that puts them apart from meningiomas of the medial sphenoid wing<sup>[2,3]</sup>. Therefore anterior clinoidal meningiomas should be considered a separate clinical entity<sup>[2-4]</sup>.

Visual loss is the very common presenting symptom in these tumors<sup>[5]</sup>. The characteristic onset usually occurs with unilateral failure of vision loss associated with primary optic atrophy<sup>[5,6]</sup>. In some patients, loss of vision may extend to the uninvolved eye<sup>[7]</sup>. Visual field defects in the form of concentric narrowing of the visual field, central scotoma, and temporal hemianopsia can be seen in patients; however, the extent depends on the degree of the involvement of the chiasm and the optic nerves<sup>[6,8]</sup>. Headache is another most common symptom in these tumors, generally over the orbital or retroorbital field<sup>[9]</sup>. Although the visual problems are the major cause of presenting symptoms in anterior clinoidal meningiomas (45.3%, 53.3%, 58% in some series)<sup>[9]</sup>, this rate is not as high as in other meningiomas located in the neighboring region such as tuberculum sellae meningiomas (75.9%-100.0%). The growth potential of the latter tumors may result in early impingement on the optic apparatus. However, for the anterior clinoidal meningiomas, this is true only for Al-Mefty group III tumors that encroach on the optic nerve at the optic foramen<sup>[10]</sup>.

Several classification schemes have been proposed as methods for predicting surgical outcome. Al-Mefty's classification of clinoidal meningiomas is based on advances in microsurgical anatomy and has been widely accepted<sup>[11,12]</sup>. This scheme considers the origin of the tumor and its invasion pattern in the region of the clinoid process as indicators of resectability. It differentiates among Group I, lower clinoidal meningiomas (no arachnoidal dissection plane between the internal carotid artery (ICA) and tumor); Group II, distal or lateral clinoidal meningiomas (which do have an arachnoidal plane between ICA and tumor) and Group III, meningiomas that originate at the optic foramen. In Group III tumors, the arachnoidal membrane is present between the ICA and the tumor but may be absent between the optic nerve and the tumor [Figure 1]<sup>[12]</sup>.

Because anterior clinoidal meningiomas tend to grow upward, true invasion of the cavernous sinus is very rare. The current literature on anterior clinoidal meningiomas documents a very wide range of cavernous sinus invasion rates, ranging from 0 to 44.1%. We believe that this variability is, in part, a reflection of the lack of objective and universal nomenclature among authors. This problem has led to some medial sphenoid wing meningiomas being analyzed as part of the clinoidal group<sup>[13]</sup>.

Based on the new classification, the coronal diameter of the tumor is combined with the classical Al-Mefty group designation. Each tumor was graded (typed) with a numerical number denoting the Al-Mefty grouping and a capital letter representing the tumor size on coronal section<sup>[14]</sup>. A tumor was defined as type A if it was measured less than 2 cm, type B if it was 2 to 4 cm, and type C (giant) if it was greater than 4 cm. These designations were applied to each group in the current Al-Mefty classification [Figure 1]<sup>[12]</sup>.

Many surgeons used various skull-base approaches with or without intra- or extradural removal of anterior clinoid for resecting these challenging tumors. The recent articles by Al-Mefty<sup>[14]</sup>, Lee *et al.*<sup>[15,16]</sup> described a cranial base technique that is a modification of the original "Dolenc approach" and involves extradural clinoidectomy, removal of the roof of the optic canal, and opening of the optic nerve sheath. Mathiesen *et al.*<sup>[17]</sup>

Anterior clinoidal meningioma classification		
Al-Mefty group	Suprasellar extension	
Group I	A	B
Group II		C
Group III	≤ 2 cm	2-4 cm
		≥ 4 cm (giant)

**Figure 1.** Classification system for anterior clinoidal meningiomas: the coronal size of the tumor is categorized and each category represents a subdivision of each Al-Mefty group classification (cited with permission from <https://clinicalgate.com/anterior-clinoidal-meningiomas/>)

also stated that, to achieve or ensure better visual function, it is best to use an extradural approach with drilling of the anterior clinoid and removal of the roof of the optic canal before any intradural steps are performed.

## METHODS

All patients were investigated preoperatively with coronal computed tomography (CT) and triplanar contrast-enhanced magnetic resonance imaging of brain. Digital subtraction angiography and CT angiogram were done in some cases to delineate the anatomy of the cerebral circulation, encasement of major vessels, arterial displacement, and blood supply. Visual testing that included determination of visual acuity, visual field and fundal photography was performed preoperatively and postoperatively in all cases. Al-Mefty<sup>[14]</sup> and Lee *et al.*<sup>[18]</sup> exclusively used the orbitocranial approach and stated its advantages as: the shortest distance to tumor, suitability for surgical attack via multiple routes, and early interception of the tumor's blood supply through the sphenoid ridge.

### Surgical technique

The patients were positioned in supine with head turned contralesional 30-degree angle, fixed with three pin head fixators. Standard pterional craniotomy was done in all cases. Following pterional craniotomy extradural anterior clinoidectomy was done either by high speed drill or bone forceps [Figure 2].

Extradural identification of optic nerve and clinoidal carotid artery was done. Curvilinear durotomy over the tumor was done which was extended to the optic nerve or carotid artery in a T fashion to enhance close proximity of optic nerve, carotid artery and tumor. Gentle debulking of the tumor following cautery of tumor's dural attachment was done. Peripheral dissecting of tumor from the brain parenchyma was done following bipolar cautery of the feeding vessels to end from brain. Finally, delicate dissection from middle cerebral artery (MCA), anterior cerebral artery (ACA), posterior communicating artery (PCOM), carotid bifurcation area, optic nerve and chiasm was done. Dural closure was done with or without graft, bone and wound was closed in multiple layers.

## RESULTS

We operated upon 10 cases of clinoidal meningioma. Among them 3 were male and 7 were female [Table 1].

The ages of patients ranged from 21-60 years. The mean age was  $45 \pm 13.12$  years [Table 2].

**Table 1. The distribution of patients by gender (n = 10)**

Gender	No. of patients	Percentage
Male	3	30.0%
Female	7	70.0%
Total	10	100.0%

**Table 2. The age of the patients (n = 10)**

Age group (in years)	No. of patients	Percentage
21-30	1	10.0%
31-40	1	10.0%
41-50	7	70.0%
51-60	1	10.0%
Total	10	100.0%
Mean age	45 ± 13.12 years	

**Table 3. The extent of tumor removal (n = 10)**

Extent of tumor removal	No. of patient	Percentage
Gross total	5	50.0%
Near total	5	50.0%
Total	10	100.0%

**Table 4. Complications (n = 10)**

Name of complication	No. of patients	Percentage
Hematoma	1	10.0%
Internal carotid artery injured	1	10.0%
Recurrence	1	10.0%
No complication	7	70.0%
Total	10	100.0%

**Table 5. Visual outcome (n = 10)**

Functional outcome	No. of patients	Percentage
Improved	7	70.0%
Static	2	20.0%
Deteriorated	1	10.0%
Total	10	100.0%

Gross total removal was achieved in 5 (50.0%) cases [Figures 3 and 4], and near total in 5 cases (50.0%) [Table 3].

In our study most of the patients had limited complications. Only one patient had complicated by hematoma and another, patient had ICA injury [Table 4].

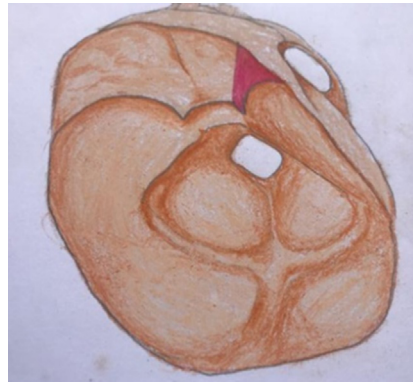
In our series, we found visual improvement in 7 cases (70.0%) [Figures 5 and 6], static in 2 cases (20.0%), and deterioration in 1 case (10.0%) [Table 5].

Papilledema also improved significantly after removal of tumors as shown in Figures 7 and 8.

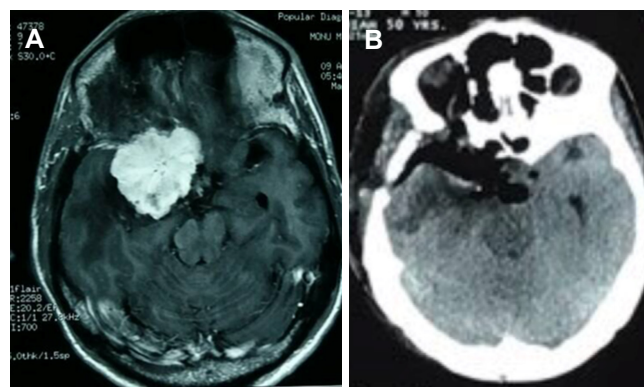
The profile of the patients with clinoidal meningioma is shown in Table 6.

## DISCUSSION

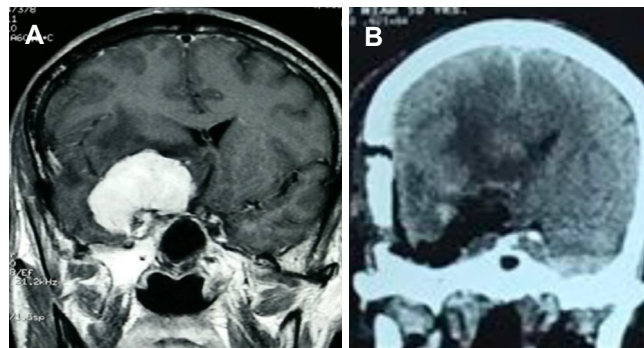
The traditional approach for the resection of anterior clinoidal meningiomas is the pterional intradural



**Figure 2.** Following craniotomy shows removal of anterior clinoid process and unroofing of optic foramen



**Figure 3.** (A) Preoperative axial contrast magnetic resonance imaging of brain shows homogeneously brilliant contrast enhancing benign tumor; (B) postoperative axial contrast computed tomography scan of brain shows complete removal of the tumor



**Figure 4.** (A) Preoperative coronal contrast magnetic resonance imaging of brain shows homogeneously brilliant contrast enhancing tumor; (B) postoperative computed tomography scan shows removal of anterior clinoid process with complete removal of the tumor

transsylvian approach, which begins with splitting the sylvian fissure, releasing cerebrospinal fluid, and debulking the tumor, and then proceeding with peripheral tumor dissection from neurovascular structures<sup>[19-21]</sup>. In this series, we present our experience using the pterional craniotomy with extradural drilling of anterior clinoid process. The surgical challenges are associated with these giant tumors from their size, difficult location, as well as the dissection, and preservation of the critical neurovascular structures like the cavernous sinus, cranial nerves, ICA, ACA, MCA and anterior choroidal artery that they inevitably involve or encase<sup>[22-24]</sup>. These challenges are increased by tensed brain, secondary edema, and tumor mass effect<sup>[18,25]</sup>.



**Table 6. Profile of the patients with clinoidal meningioma**

Sl. No.	Age (years)/gender	Symptoms	Size of tumor (cm <sup>3</sup> )	Extent of tumor removal	Name of operation	Complication	Visual outcome
1	50/F	Headache	6*6*6	Gross total	Pterional craniotomy and anterior clinoidectomy	Hematoma	Static
2	45/F	Headache	8*6*5	Gross total	Pterional craniotomy and anterior clinoidectomy	Internal carotid artery injured, 3rd nerve palsy	Deteriorated
3	50/F	Headache	6*6*5	Gross total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved
4	25/F	Lt eye blind	6*5*5	Near total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved
5	58/M	Headache	5*4*5	Near total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved
6	36/F	Headache	5*4*5	Near total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved
7	42/F	Headache	4*5*4	Near total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved
8	45/F	Headache	4*5*5	Gross total	Pterional craniotomy and anterior clinoidectomy	Recurrence	Static
9	45/M	Headache	6*5*4	Gross total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved
10	47/M	Headache	6*5*4	Near total	Pterional craniotomy and anterior clinoidectomy	Nil	Improved

The concept of an extradural approach to skull base tumors is not new. After the initial work of Dolenc<sup>[11]</sup>, the technique he introduced as an approach to the cavernous sinus evolved in the hands of other surgeons for removal of medial sphenoid wing/clinoidal meningiomas<sup>[26]</sup>.

There are 2 main challenges in the safe removal of giant tumors: (1) how to safely locate the important arteries and the optic apparatus inside these giant tumors, and (2) how to avoid damage to tensed brain during approach, dissection, and tumor removal<sup>[27]</sup>.

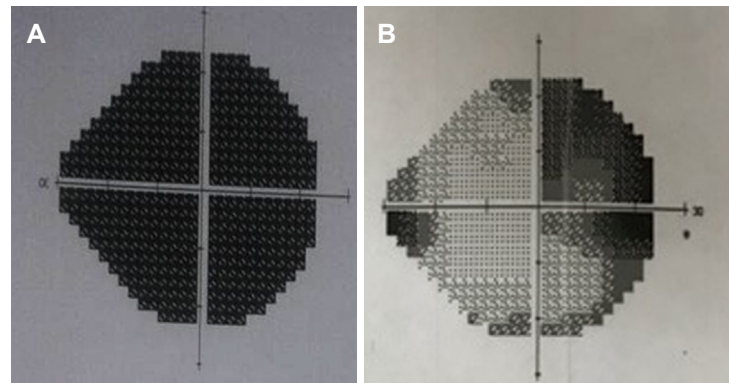
We presumed that the best way to avoid damaging the ICA and optic nerve was to locate and dissect them in areas in which the anatomy remains relatively normal, with minimal distortion from the tumor. Extradural clinoidectomy solves this problem<sup>[28,29]</sup>.

The extradural skull base approach that was used in our patients is similar to the technique reported by Lee *et al.*<sup>[18]</sup> with some modifications. They reported on 15 patients with somewhat smaller anterior clinoidal meningiomas (mean diameter 3.7 cm), including 8 patients presenting with preoperative visual deficits. After surgery, vision improved in 75% cases. This good result could be related to their extradural approach and early optic nerve decompression.

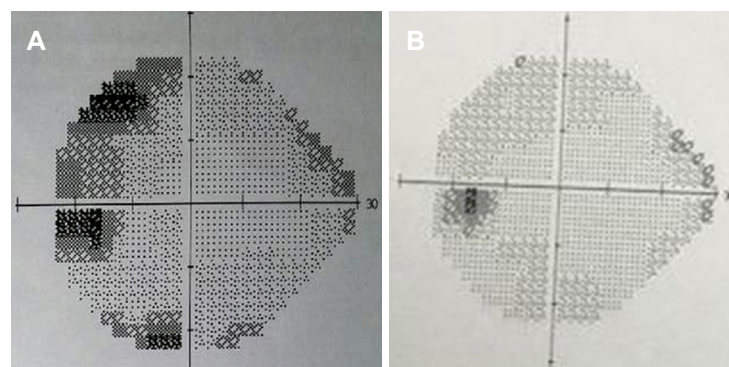
In our series, we achieved visual improvement in 7 cases (70.0%), static in 2 cases (20.0%) and deterioration in 1 case (10.0%).

In another study, 20 patients with preoperative visual deficits due to giant medial sphenoid wing meningiomas, Behari *et al.*<sup>[5]</sup> attained visual improvement in 3 patients and stable visual function in 11; 5 patients experienced deterioration of vision in the ipsilateral eye at a mean of 17.6-month follow-up. The majority of patients in the series of Behari *et al.*<sup>[5]</sup> had stable vision after surgery, while in our experience most patients' vision improved. The team of Behari *et al.*<sup>[5]</sup> performed early extradural unroofing of the optic canal and optic nerve decompression in only 15.8% of their patients, whereas we used this technique in all cases.

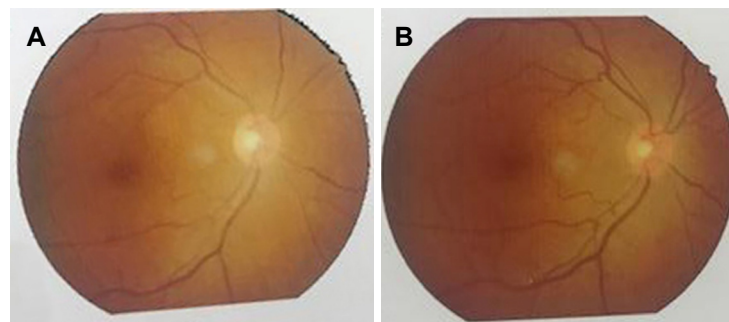
In a series of 35 patients with medial sphenoid wing meningiomas (mean diameter 4.5 cm), Russell and



**Figure 5.** Humphrey visual field test. (A) Preoperative VF (right eye) with total blindness; (B) postoperative VF (right eye) with significant visual improvement

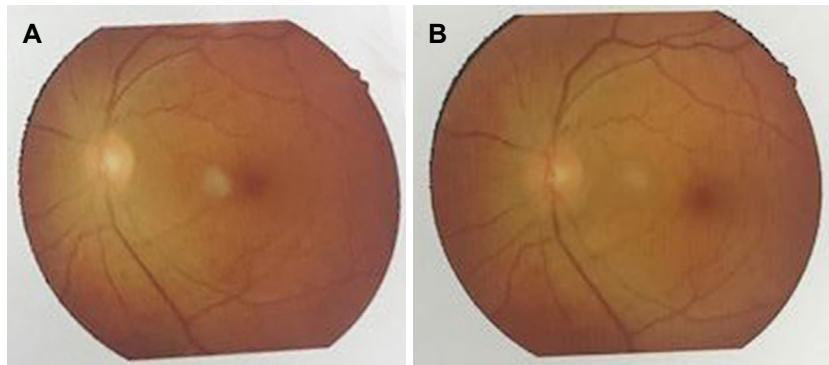


**Figure 6.** Humphrey visual field test. (A) Preoperative VF (left eye) with upper temporal field; (B) postoperative VF (left eye) with significant visual improvement defect



**Figure 7.** (A) Preoperative fundal photography (right eye) with primary optic atrophy; (B) postoperative fundal photography (right eye) with slight improvement

Benjamin<sup>[30]</sup> reported visual improvement in 73%, stable vision in 20%, and deterioration in 7% of patients who had visual loss before surgery. Pamir *et al.*<sup>[31]</sup> also reported improvements in visual function in the majority of 43 patients with anterior clinoidal meningiomas with a mean tumor diameter of 3.35 cm, including 16 patients with tumors larger than 4 cm in diameter. Among 26 patients who had preoperative visual deficits, 84.6% improved and 15.4% remained stable. The Russell and Pamir teams used an intradural approach. In our series, there were no deaths, in comparison with 5%-15.4% mortality in some recent series. Tomasello *et al.*<sup>[22]</sup>, whose patients had a mean tumor diameter of 5.7 cm and of whom 77% underwent



**Figure 8.** (A) Preoperative fundal photography (left eye) with normal findings; (B) postoperative fundal photography (left eye) with static findings

gross total resection, reported the highest mortality rate in patients treated via the conventional pterional intradural transsylvian approach. Behari *et al.*<sup>[5]</sup> reported 5% mortality.

In our series, 3 patients (13.6%) had postoperative hemiparesis. In 1 patient, the hemiparesis was caused by intracranial hematoma due to intraoperative hematoma. In other 2 patients, the deficit was most likely due to manipulation of perforating vessels encased by the tumor. Goel *et al.*<sup>[12]</sup> reported postoperative hemiplegia in 6.6% of patients. Behari *et al.*<sup>[5]</sup> reported 10% temporary and 5% permanent hemiparesis, and Tomasello *et al.*<sup>[22]</sup> reported 7.6% hemiparesis. The encasement of small perforating vessels in clinoidal/medial sphenoid wing meningiomas is a serious problem. Injury to small perforating arteries during tumor resection is a known cause of neurological deterioration, even when the large parent vessels are well preserved<sup>[32,33]</sup>.

In conclusion, giant anterior clinoidal meningiomas are very challenging tumors. We prefer an extradural skull base approach to the tumor, including extradural unroofing of the optic canal, extradural clinoidectomy, early optic nerve decompression, and early identification and control of the clinoidal carotid artery followed by removal of the remaining tumor. This technique has provided a good extent of resection, as well as a good visual and clinical outcome.

## DECLARATIONS

### Authors' contributions

Conception, diagnosis and design, radiology diagnosis and final approval of manuscript: Alam S, Chaurasia BK

Manuscript preparation: Alam S, Wakil Uddin AN, Majumder MR, Shalike N, Chowdhury D

Literature search: Wakil Uddin AN, Majumder MR, Shalike N, Chaurasia BK, Khan AH

Technical revision, manuscript editing and revision: Alam S, Shalike N, Ansari A, Barua KK

### Data source and availability

The data can be obtained from the computer database of Department of Neurosurgery, Bangabandhu Sheikh Mujib Medical University, Shahbagh, Dhaka-1000, Bangladesh, but it cannot be explored online.

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### Conflicts of interest

There are no conflicts of interest.

## Patient consent

Patients' consent was obtained from the patients.

## Ethics approval

Our research proposal has been reviewed and approved by the Institutional Review Board (I.R.B.), Bangabandhu Sheikh Mujib Medical University in its 106th meeting held on 15 May 2013. Number of ethics approval is No. BSMMU/2013/2334.

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Commentary

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# Commentary on interactions between neurotropic pathogens, neuroinflammatory pathways, and autophagic neural cell death

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Autophagy is a fundamental regulatory cellular mechanism, which enables cells to survive after a checked clearance of damaged organelles and proteins and a recycle of necessary molecules like amino acids and fatty acids to maintain homeostasis<sup>[1]</sup>. There are a lot of factors able to influence autophagy in the states of health and disease. In the excellent review by Sahu *et al.*<sup>[2]</sup>, the role of autophagy, its mechanisms, and its protective role against the attack of pathogens are well described. However, even more controversial aspects of autophagy are addressed: first some pathogens, such as herpes simplex viruses, coxsackievirus, listeria monocytogenes have developed strategies to circumvent autophagy-dependent activation of host immune response, then some bacteria have the ability to modify the gene transcriptional level of the autophagic process, both in terms of downgrading of autophagy-related genes, as in the case of *Yersinia enterocolitica* and *Francisella tularensis*<sup>[3]</sup>, and in terms of up regulation of autophagy-related genes: this mechanism favors a prolonged inflammation at the infection site and results in further injury to surrounding healthy tissues, causing brain matter degeneration.

The proposed hypothesis that prolonged infections of the central nervous system (CNS) by neurotropic pathogens, if associated with underlying conditions, might play a role in the pathogenesis of neurodegenerative diseases and the connections between autophagy dysregulation and neurodegeneration are subjects of great interest in literature.

Recent studies have demonstrated an intrinsic connection between selective autophagy impairment and neurodegenerative diseases, including Alzheimer's disease (AD)<sup>[4-7]</sup>, Parkinson's disease (PD)<sup>[8-10]</sup>, Huntington's



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disease (HD)<sup>[11-14]</sup>, amyotrophic lateral sclerosis (ALS)<sup>[15-17]</sup>, and multiple sclerosis (MS)<sup>[1,18-23]</sup>.

AD is a neurodegenerative chronic disorder characterized by a progressive cognitive decline; its main etiologic mechanisms are: deposition of extracellular amyloid- $\beta$  plaques, synaptic loss and evidence of intracellular neurofibrillary inclusions with hyper-phosphorylated tau protein.

Even if autophagy plays a complex role in AD, which needs to be further characterized, the connection between this disease and autophagy dysregulation has been already demonstrated: a pathological accumulation of autophagosomes in neocortical of AD patients has been proved<sup>[5]</sup> and the mutations of presenilin-1, which are related to AD forms with early-onset, cause a block of the autophagy flux in fibroblasts in these patients<sup>[24,25]</sup>. On the other hand, other investigators suggest that early microglial accumulation in AD delays disease progression by promoting clearance of A $\beta$  before formation of senile plaques, therefore having a protective role<sup>[25]</sup>. The ability of microglia to clear A $\beta$  may decrease with age and progression of AD pathology<sup>[25]</sup>.

In PD one of the pathologic features is the presence of Lewy bodies, abnormal deposits of a protein called  $\alpha$ -synuclein: this fact is suggestive of defects in intracellular protein clearance mechanisms and autophagic process dysregulation<sup>[26]</sup>.

HD is caused by expansion of a CAG trinucleotide repeat in the first exon of the huntingtin gene that encodes the mutant huntingtin: this protein is highly expressed in neurons and participates in many cellular functions, including vesicle and organelle transport and autophagy<sup>[13,27]</sup>. Mutant huntingtin interferes with the correct autophagic function; therapeutic strategies that improve the clearance of this mutant protein by autophagy reduce neuronal toxicity<sup>[14]</sup>.

Recent genetic evidence has demonstrated that mutations in autophagy-regulatory genes can result in the occurrence of ALS<sup>[15-17]</sup>.

MS is a chronic immune-cell-mediated disease characterized by the presence of auto-reactive T cells target the myelin sheath in the CNS<sup>[18]</sup>, leading to inflammation, demyelination and neuronal function impairment. In patients affected by MS, several autophagy-related genes (e.g., ATG-16L2, ATG-9A, and ULK-1) are overexpressed<sup>[1]</sup>, therefore the hypothesis is that over-activation of autophagy may contribute to auto-reactive T lymphocyte survival<sup>[18]</sup>.

Given the pivotal roles of the autophagy in these neurodegenerative diseases, the targeting of some key pathways of the inflammatory process provides new insights into the diagnosis and the modulation of this process represents a new therapeutic strategy for neuroprotection<sup>[1]</sup>.

The activated microglia plays a major role in chronic neuroinflammation, causing long-term cerebral damage by inducing autoimmune reaction, and is observed in various CNS diseases such as stroke, MS, ALS, AD and PD<sup>[28]</sup>. *In vivo* imaging of activated microglia can provide a non-invasive and reliable detection of early and localized neuroinflammation processes, thanks to the availability of several neuroimaging modalities<sup>[29]</sup>. Positron emission tomography (PET) is an imaging technique that can characterize measure and visualize the biological processes at the molecular levels in the body<sup>[28]</sup>.

In addition to the standard glucose metabolism [<sup>18</sup>F fluorodeoxyglucose (<sup>18</sup>F FDG) PET], a variety of targets for inflammation imaging have been recently discovered, and the corresponding PET tracers showed high levels of accuracy and are considered superior to FDG for imaging inflammation<sup>[30]</sup>. The most studied neuroinflammation related targets include translocator protein (TSPO), type 2 cannabinoid receptor (CB2R), and cyclooxygenase (COX)<sup>[31]</sup>.

TSPO is an 18 kDa translocator protein located on the outer mitochondrial membrane. It is only minimally expressed in the healthy human brain, but highly expressed in macrophages, neutrophils, lymphocytes, astrocytes and microglia<sup>[32]</sup>. TSPO expression is highly up-regulated during the microglia activation process in several acute and degenerative disorders, including AD, PD, ALS, MS, and HD; therefore, TSPO is considered a promising target for PET imaging of neuroinflammation and has already undergone clinical application<sup>[30]</sup>. TSPO PET imaging, using various PET tracers binding to TSPO<sup>[33]</sup>, has been used for both improving the knowledge regarding the role of neuroinflammation in CNS diseases and to assess the efficacy of novel anti-inflammatory therapeutic strategies. In a rat brain AD model elevated TSPO levels were found in tau-rich hippocampus and entorhinal cortex region of the brain and there was a constant increase of tracer uptake in the brain region with the progression of AD<sup>[34]</sup>. In PD patients, microglia activation could be detected via TSPO PET at the early phase of the disease, and its activity is correlated with the development of dementia in PD<sup>[35]</sup>.

Cannabinoid receptors are a family of G-protein-coupled receptors composed at least by two subtypes: type 1 and 2. While type 1 is abundantly expressed in the brain, type 2, which is the inducible isoform, is detectable just in microglial cells, in human fetal astrocytes, and in human cerebral microvascular endothelial cells<sup>[36]</sup>. Several studies reported an up-regulation of CB2R on activated microglial cells in pathological conditions, including MS, ALS, PD, or AD<sup>[37]</sup>. The activation of CB2R by CB2 agonists is found to be effective in reducing neurodegeneration in HD and ALS transgenic mouse model, thanks to the decrease of microglial activation, mediated by the release inhibition of neurotoxic factors and by the decrease of neuronal cell damage<sup>[38]</sup>. Therapeutic modulation of CB2R could be a promising treatment for neuropathogenic disorders characterized by a neuroinflammatory component. Over the past years, several CB2R selective ligands have been developed and labeled with radioisotopes for PET, including pyrazole derivatives, indole derivatives, and quinoline derivatives<sup>[30]</sup>. The first *in vivo* PET of brain CB2R showed a significant increase in tracer uptake in all brain regions in mice with lipopolysaccharide induced neuroinflammation; in this study, tracer uptake could be blocked by a CB2R selective ligand, thus indicating the specificity of the tracer accumulation<sup>[39]</sup>. New synthesized radiotracers are under investigations in the preclinical settings and offer promising opportunities for imaging CB2R expression in the future<sup>[31]</sup>.

Cyclooxygenase (COX) is an enzyme producing important biological mediators mainly expressed in neuroinflammation in connection with neurodegenerative diseases. Three COX subtypes (COX-1, 2, and 3) have been identified. COX-2 is the inflammatory inducible enzyme form of this family and its expression in brain has been associated with neurodegenerative processes of several acute and chronic diseases<sup>[40]</sup>.

COX-2 has been identified as a molecular target of interest for pharmacological design of selective ligands for both therapy and molecular imaging. Celecoxib is an important and widely used anti-inflammatory drug that inhibits selectively COX-2 to treat various inflammatory diseases. In order to image neuroinflammation, Celecoxib and other COX inhibitors were manipulated using imaging tracers (18F and 11C): these attempts have not yet shown significant results due to the absence of specific bindings, sensitive enough to inflammatory foci<sup>[30]</sup>. On the other hand, a study of a rat skin model of inflammation showed significant uptake in COX-2 targeted micro PET/ computed tomography (CT) imaging of a mouse paw inflammation induced by carrageenan<sup>[41]</sup>.

Despite the fact that the administration of selective COX-2 inhibitors has been shown to attenuate brain inflammatory reactions, protecting neurons against neurodegeneration in AD patients<sup>[42]</sup>, the evidence of a direct role of COX-2 in neurodegenerative events is still controversial: recent data demonstrates that COX-1, classically viewed as the homeostatic isoform, is involved in brain injury induced by pro-inflammatory stimuli<sup>[43]</sup>.

Moreover, thanks to the recent discover of high specificity of cellular expression of COX-1 within microglia during acute neuroinflammatory process, the development of a selective COX-1 imaging probe has regained

interest and it showed a better capacity to be depicted by PET *in vivo*<sup>[44]</sup>; in an animal model of AD, PET images clearly showed COX-1 expression in activated microglia during the formation of amyloid plaques<sup>[41]</sup>. PET imaging of COX-1, according to these preclinical studies, could be a promising approach for monitoring activated microglia in CNS diseases such as AD<sup>[31]</sup>.

Neuroinflammation plays a crucial role in the development of neurodegenerative disorders. The information extracted from molecular imaging of inflammation in the CNS could be fundamental for understanding the causes and effects of this entity, its relationship with various pathogens and other stimuli, and could be crucial in disease diagnosis, prognosis and in future therapies response monitoring. More specific biomarkers have to be identified and more sensitive imaging probes must be developed to improve visualization and quantification of the inflammatory processes and to better understand and characterize the interactions between neurotropic pathogens, neuroinflammatory pathways, and autophagic neural cell death and the CNS disorders progression.

## DECLARATIONS

### Authors' contributions

Worked on the first part of the article: Cellina M

Worked on the part of new perspectives: Orsi M

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Commentary

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# Commentary on “Depression severity and its predictors among multiple sclerosis patients in Saudi Arabia: a cross-sectional study”

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Multiple sclerosis (MS) is looked upon as chronic autoimmune disease. Current therapeutic research focus on the prevention of relapses in association with morphological magnet resonance imaging (MRI) parameters. This paper again describes that depression is one of the most common symptoms in MS. Components of depression for manifestation in MS are exogenous, such as disability, or endogenous, i.e., due to localisation of lesions, which predispose for onset of depression. This paper points out that depression and related neuropsychiatric symptoms, i.e., apathy and fatigue, should not be underestimated in the clinical maintenance of MS patients. This paper supports the view, that further research is warranted beyond past and future ongoing trials on cognitive deficits, which frequently disregard the impact of apathy and fatigue on standardised neuropsychological testing in association with chronic intake immune system modulating compounds. Therefore it is promising that efforts are undertaken on standardisation of neuropsychological assessment tools, i.e. for cognition, in trials<sup>[1]</sup>, whereas the MiniMental State Examination score have a considerable bias by the educational level of the patient. Clinicians repeatedly point out, that non-cognition related signs are often essential limiting for quality of life. As a result, they investigate the efficacy of already available compounds often in observational or naturalistic small trials, like in this paper. Clinicians point out that non-cognition related signs are often essential limiting for quality of life. As a result, they investigate the efficacy of already available compounds often in observational or naturalistic small trials. These outcomes are frequently considered as less essential by the authorities driven evidence-based-medicine classification of trials. One must consider that most of the used assessment instruments are not objective. They are biased by the attitude and habits of the investigator. One underestimates that the rating situation and the stress for the



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patient often cause an insufficient appraisal of the tested compound.

Nevertheless, it is worth to mention that certain drugs, such as interferons with their flu-like side effect profile and their more frequent application rate, support onset of certain neuropsychiatric symptoms in contrast to glatiramer acetate or compounds with distinct less frequent intake, such as natalizumab, ocrelizumab or cladribin. In this respect, outcomes of Table 6 are of interest in combination with the discussion on the severity of depression in relation to the applied medications. Here the authors conclude that drug side effects may account for the found differences between treatments. This is an important aspect, which leads the way to select medications with a need for less frequent intake, i.e., cladribine or ocrelizumab, in the future. Thus, this paper also emphasizes by circumstantial evidence that not only reduction of the annual relapse rate or MRI changes are important but also the kind of MS treatment for prevention of relapses. Another point is the individually different necessary symptomatic therapy with spasticity ameliorating compounds or cannabis like compounds. Nearly all of them induce fatigue. Moreover, dosing depends on concomitant factors, i.e., body weight, severity of spasticity in relation to the localisation and size of lesions. Therefore, this trial also underlines again that (1) MS therapy is complex, (2) asks for a patient tailored regime particularly in the more advanced stages of the disease, and (3) maintenance of MS patients often faces the additional appearance of various kinds of non-motor symptoms, i.e., depression. There is also hysteria on safety. In the real world, clinical researchers underline the importance of the so-called nocebo-effect. This means that patient experiences a side effect once being informed on its potential occurrence<sup>[2,3]</sup>. In the clinical research scenario, the side effect profile and the tolerability of a tested immune system modulating compound appears to have more or at least the same importance than its efficacy. In clinical practice however, the application of a compound is often the result of a careful benefit-risk evaluation performed by the prescribing physician and the more and more well informed, mature patient. It is more important to select a therapy for the modulation of the immune system, which is well tolerated and accepted by the individual patients. This also increases the adherence to compound. Particularly, compliance is an important issue in the maintenance of MS patients. Missing adherence may also contribute or trigger the Immune-reconstitution inflammatory syndrome. If it occurs, it will may in turn weaken the confidence of the patient and the physician in the applied compound<sup>[4-6]</sup>.

In contrast, the current artificial clinical study world mostly only focus on relapse prevention and MRI findings. The fancy translational approach to test compounds, which were successful in experimental autoimmune encephalitis models with their focus on relapse prevention by modulation of the immune system only, looks promising, but do not reflect all the therapeutic challenges of clinical practice. The limitation of these experimental models and thus the performed experimental investigations is the focus on the immune system. These models often only mirror mechanisms of neuronal dying based on immunological mechanisms modulated by B- or T-cells. Thus, experimental research neglects that chronic neuroinflammation and associated neurodegeneration may also cause further consequences, such as psychopathological features and personality changes. The register trials often use quality of life scales, which disregard the individually varying, existing capacity of the human brain to compensate these neuropsychiatric events for certain intervals before the clinical onset of initial mild and unspecific symptoms. This so-called “neuroplasticity” phenomenon may also impact the rate of progression and thus differs in an individual different manner. In summary, this heterogeneous and individual different disease progression in combination with relative short trial periods may also contribute to a failure of trials on disease modification, particularly in progressive MS. Mortality or increase of life expectance, caregiver burden or delay of transfer to nursing homes may represent more robust clinical endpoints in terms of disease modification in comparison to the mostly applied expanded disability status scale score or the artificial conversion endpoints from relapse remitting to progressive MS. One must admit that the aforementioned suggested, alternative endpoints would demand longer study durations particularly in the real world, as suggested in this paper. However, the real world finally determines the value of treatment and the efficacy of drugs.

## DECLARATIONS

### Authors' contributions

Müller T contributed solely to the paper.

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Original Article

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# Shifting paradigm in brain abscess management at tertiary care centre in Nepal

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## Abstract

**Aim:** Brain abscess is a challenging clinical entity with substantial high case fatality rates despite significant advances in imaging techniques, laboratory modalities, surgical interventions, and antimicrobial treatment. Otogenic and cardiogenic sources are among the most common. Classic clinical presentation is seen in very few cases only. Burr hole with aspiration works well with good clinical outcomes. Control of primary source in cases of ear infection in the single setting results in good outcomes, reduces for additional surgery, and decreases the duration of hospital stay.

**Methods:** This is prospective observational study conducted at Institute of Medicine, Tribhuvan University Teaching Hospital, Kathmandu, Nepal over the period of two and a half years (from September 2014 to March 2017). We analyzed the demographic profile, management strategies and outcome of these cases diagnosed with brain abscess using Microsoft Excel 2007.

**Results:** A total of 51 cases were undertaken for surgical management. There were 35 males and 16 females with the male to female ratio of 2.18:1. The mean age of the study population was 16.76 years with age range from 4 months to 60 years. Otogenic source was the most common. Temporal lobe was the most common abscess location. Headache was the most common clinical presentation and was seen in 86.27% of the study population. All cases were initially managed with burrhole and aspiration of the abscess. Only 3.92% ( $n = 2$ ) of cases subsequently required surgical excision of the abscess wall. Only 11.76 % ( $n = 6$ ) of the cases required multiple aspiration. Only 19.61% ( $n = 10$ ) showed positive culture. *Pseudomonas aeruginosa* and *E. coli* were the most common organisms grown. Mortality rate among the study group was 3.92%.



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**Conclusion:** With the advent of modern technology in neuroimaging, mortality due to brain abscess has significantly decreased. Joint involvement of the otorhinolaryngology team and efforts in addressing the primary source have further helped in improving outcomes in cases of otogenic brain abscess. Hence, source control is of paramount importance in managing the brain abscess.

**Keywords:** Abscess, otogenic brain abscess, tubercular abscess

## INTRODUCTION

Though the incidence of brain abscesses is as low as 2% in the western world, it accounts for up to 8% of intracranial masses in developing countries<sup>[1,2]</sup>. There is a paucity of data about the exact incidence of brain abscess in Nepal. Despite recent advances in imaging techniques, laboratory modalities, surgical interventions and antimicrobial treatment, brain abscess still remains a challenging clinical entity with substantially high case fatality rates. This is attributable to changes in epidemiology, clinical spectrum, predisposing factors and the prevalence of implicated bacterial pathogens. Brain abscess contributes to high mortality, and even more so in immune compromised patients. Different organisms have been implicated in its etiology comprising of bacteria, mycobacterium, fungi, parasites (protozoa and helminthes) and cryptogenic<sup>[3]</sup>. Brain abscess formation may occur after neurosurgical procedures or head trauma. Brain abscesses due to contiguous spread from parameningeal foci of infection (e.g., the middle ears, mastoids, and sinuses) are common in developing countries. Many of the patients with brain abscess have concomitant ear infection and/or cardiac problems, especially cyanotic heart disease. Formation of brain abscess after penetrating head injuries and following cranial surgery is also common. The present study presents the demographic profile, surgical management, microbiology and outcomes at our centre.

## METHODS

This is a prospective observational study conducted over the period of two and half years (from September 2014 to March 2017) at Institute of Medicine (IOM), Tribhuvan University Teaching Hospital Kathmandu, Nepal. Data regarding patient characteristics, clinical profile, etiology, microbiology profile, management algorithm and complications were collected and analyzed. All cases with brain abscess admitted to IOM were included in the study. Individuals with brain abscess operatively managed in other hospitals and referred to our centre postoperatively were excluded from the study. Clinical findings at presentation were noted and subsequently contrast enhanced computed tomography (CECT) was done in all cases. High resolution computed tomography (HRCT) of the involved ear was also performed in indicated cases. Initially, hyperosmolar therapy (mannitol-1 gm/kg/ IV dose q 8 hourly and dexamethasone 1 - 4 mg IV q 8 hourly depending up on the weight of patient) was instituted until surgical intervention was completed and steroids were tapered. Antiepileptic medications were instituted for all supratentorial abscesses for a minimum of 1 month and continued in patients with seizures for a period of 2 years. Burr hole and drainage is the standard procedure performed in our centre after marking the operative site on CT scan. Craniotomy and abscess cavity excision were required in very few cases. After drainage, obtained samples were sent for Gram stain, culture and sensitivity. Anaerobic culture was not done due to resource constraints. Patients were treated with initial empirical triple broad spectrum antibiotics which included vancomycin 15 mg/kg IV 8 hourly, ceftriaxone 25 mg/kg IV in two divided doses and metronidazole 15 mg/kg IV in 3 divided doses. Narrowing of antibiotics was made accordingly and continued for 6 weeks if culture sensitivity was positive for a specific organism. Follow-up head CT was performed in 1 week and every 2 weeks thereafter. Head CT was performed in between if indicated by alteration in clinical status of the patient, i.e. drop in Glasgow Coma Scale by  $\geq 2$  points. Repeat aspiration was performed if the size of the abscess was found to be increasing or clinical status was not improving. Patients were labeled as cured radiologically if there was no residual abscess cavity on CECT after 6 weeks of treatment.

**Table 1. Etiology of brain abscess in the study population**

No.	Etiology/source	Frequency (n)	Percentage (%)
1	Otogenic (chronic suppurative otitis media)	28	54.88
2	Tubercular	7	13.72
3	Cardiogenic	6	11.76
4	Animal bite	2	3.92
6	Post craniotomy	1	1.96
7	Unknown	7	13.72

**Table 2. Location of the intracranial abscess in the study population**

Intracranial suppuration	Frequency (n)	Percentage (%)
Temporal abscess	22	43.12
Cerebellar abscess	8	15.68
Frontal abscess	7	13.72
Multiple abscess	6	11.76
Parietal abscess	4	7.84
Subdural empyema	2	3.92
Epidural abscess	1	1.96
Interhemispheric abscess	1	1.96
Total	51	100

**Table 3. Dominant clinical presentation of study population**

No.	Clinical presentation	Average duration	Frequency (n)	Percentage (%)
1	Headache	30 days	44	86.24
2	Ear discharge/ache	> 1 year	28	54.88
3	Focal neurological deficits	7 days	11	21.56
4	Vomiting	4 days	8	15.68
5	Altered sensorium	2 days	5	9.80
6	Seizure	1 day	5	9.80
7	Fever	7 days	5	9.80

## RESULTS

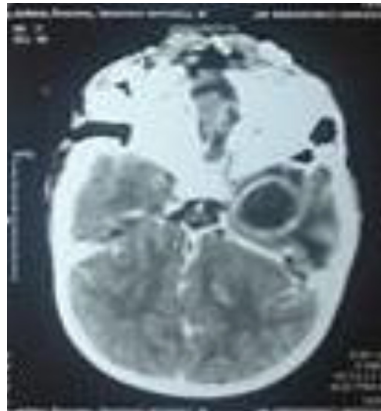
In the present study, 51 cases were undertaken for surgical management. There were 35 males and 16 females with the male to female ratio of 2.18:1. Mean age of the study population was 16.76 years, ranging from 4 months to 60 years. More than 50% of the study population ( $n = 26$ ) were  $\leq 16$  years old. Abscesses were primarily otogenic in origin [Table 1]. There were 28 cases of otogenic origin as a result of chronic suppurative otitis media (CSOM), followed by other identified sources including tubercular abscess ( $n = 7$ ), cardiogenic ( $n = 6$ ). In 13.72% ( $n = 7$ ) cases, the cause of brain abscess was not identified. Supratentorial lesion ( $n = 43$ ) was more common than infratentorial ( $n = 8$ ), and the temporal lobe was the most common site (43.13%) [Table 2].

### Clinical presentation

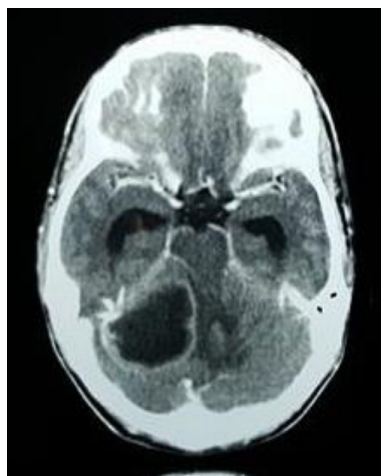
Headache and ear discharge were the most common presenting features [Table 3]. Almost all patients who could communicate were complaining of some degree of headache. Of those patients with CSOM ( $n = 44$ ), 86.27% had history of ear discharge. Focal neurological deficits were seen in 29.41% ( $n = 15$ ) of the study population. Vomiting was seen in 29.41% of the cases ( $n = 15$ ); 9.8% ( $n = 5$ ) of the study population presented to the emergency department in a state of altered sensorium and 5 patients presented with seizure. Two infants presented with poor feeding. One infant was brought in with history of infected left frontal scalp wound and was found to have exposed brain parenchyma with subdural empyema.

### Radiological evaluation

CT with contrast was the initial diagnostic modality in clinically suspected cases of brain abscess. Magnetic resonance imaging (MRI) was done in cases with subsequent diagnostic uncertainty only. The most common



**Figure 1.** Contrast enhanced computed tomography of head showing left temporal lobe abscess



**Figure 2.** Contrast enhanced computed tomography of case diagnosed with brain posterior fosa (right cerebellar) abscess with chronic suppurative otitis media

CT findings showed smooth, round/oval ring enhancing lesion with central hypo density and surrounding hypo density suggestive of edema. Peripheral enhancing lesions in the subdural and epidural spaces with or without air density were suggestive of subdural empyema and epidural abscess. Destruction of the middle ear and soft tissue density in the mastoid air cells suggested suppurative otitis media which is shown in [Figures 1 and 2](#).

### Management

All cases were managed with burrhole and aspiration of the abscess initially. Only 3.92% ( $n = 2$ ) of cases required additional surgical excision of the abscess wall. Primary control of the source was done by modified radical mastoidectomy (MRM) in patients with CSOM ( $n = 23$ ). In 5 cases of CSOM, concomitant MRM was not done at the time of abscess drainage due to poor clinical condition. These cases subsequently underwent MRM as soon as possible, and re-aspiration was done if indicated at that time. Only 11.76% ( $n = 6$ ) of the cases required multiple aspiration (2-5 times) through the same burrhole [[Table 4](#)].

### Microbiology

Most of the specimens sent for culture and sensitivity were negative for growth, presumably due to the use of preoperative antibiotics. Only 19.61% ( $n = 10$ ) showed positive culture. *Pseudomonas aeruginosa* and *E. coli* were isolated in 7 and 2 cases respectively. One case showed growth of multiple organisms including



**Table 4. Surgical procedures in the present study**

Procedure	Frequency (n)	Percentage (%)
Burrhole and aspiration with modified radical mastoidectomy	23	45.08
Burrhole and aspiration	14	27.44
Burrhole and multiple aspiration	6	11.76
Craniotomy and subdural empyema drainage	3	5.88
Craniotomy and abscess wall excision	2	3.92
Continuous abscess drainage	2	3.92
Craniotomy and epidural abscess drainage	1	1.96
Total	51	100

**Table 5. Complications observed in the study population**

No.	Complications	Frequency (n)	Percentage (%)
1	Mortality	2	3.92
2	Pyoventricle	1	1.96
3	Post modified radical mastoidectomy facial nerve palsy (grade II-V)	4	7.84
4	Pseudomeningocele	1	1.96
5	Surgical site infection	1	1.96

*staphylococcus aureus*. All cases were treated with 2 weeks of intravenous and 4 weeks of oral antibiotics (Cefpodoxime 5 mg/kg 12 hourly and Metronidazole 25 mg/kg/day in 3 divided doses) including anaerobic coverage. Some cases required 8 weeks of antibiotics. Oral versus intravenous route was determined depending on the status of the abscess cavity on repeat head CT. Culture and sensitivity for anaerobic organisms were not done in the present study due to resource constraints.

### Complications

The mortality was 3.92% ( $n = 2$ ) during the study. Major complications observed are listed in Table 5. The most common minor complication noted was thrombophlebitis likely due to prolonged use of IV antibiotics.

### Outcome

All surviving patients were followed up in outpatient clinic for at least 3 months. All of them had Glasgow outcome scale of 5/5. Surgical site infection and pseudomeningocele was resolved at the 6 week follow up visit. Facial palsy resolved in 3 cases, with residual palsy present in 1 case at follow-up cessation (3 months).

## DISCUSSION

Brain abscess comprises approximately 8% of all space occupying lesions in the brain in developing countries<sup>[1,2]</sup>. Abscess is the second most common type of intracranial complication of otogenic origin, with temporal lobe being the most common site of pathology. Clinical presentation varies among patients. The classic triad of fever, headache and focal deficit is rarely seen. Features of raised intracranial pressure with or without localizing signs require early radiological imaging to avoid inadvertent delay in management<sup>[4]</sup>. Contrast enhanced CT scan of the head is the mainstay of diagnostic modalities<sup>[5]</sup>, providing rapid means of detecting the lesion. MRI, combined with diffusion-weighted (DWI) and apparent-diffusion coefficient (ADC) images, is a valuable diagnostic tool in differentiating brain abscess from primary, cystic, or necrotic tumors with positive predictive value of 98% and negative predictive value of 92%<sup>[6]</sup>. Cultures of blood and cerebrospinal fluid identify the causative pathogen in approximately one quarter of patients. Cultures of cerebrospinal fluid may be valuable in patients with coexisting meningitis. Lumbar puncture can lead to herniation in such situations<sup>[7]</sup>.

There is no pragmatic rule for the treatment of brain abscess. Treatment of each case is individualized depending up on the location, size, and stage of abscess. The mainstay of treatment is prompt action and

initiation of antibiotics. However, surgery is imperative for the identification of the causative pathogen and for the purpose of reducing the size of the abscess. With the use of modern stereotactic neurosurgical techniques, almost any brain abscess that measures at least 1 cm in diameter is amenable to stereotactic aspiration, regardless of location. Stereotactic navigation systems can be used for abscess drainage<sup>[8]</sup>. Non-operative management of small abscess with broad spectrum antibiotics is also common. In the present study we managed the cases with burr hole and abscess aspiration. Abscess wall excision was done in cases which required additional procedures, like evacuation of subdural or epidural pathology. It was also indicated that following multiple aspirations fail to result in abscess resolution.

Another important aspect of care is managing the primary source. A number of studies have shown good results with concurrent abscess drainage and mastoidectomy in the same setting without added morbidity in cases with CSOM. They have shown low recurrence rate, though not statistically significant<sup>[9]</sup>. Other procedures being carried out include transmastoid approaches for abscess<sup>[10]</sup>.

Brain abscess is seen in 5%-18% cyanotic congenital heart diseases and these individuals are 10 times more prone to develop brain abscess than those without cyanotic heart disease. Tetralogy of Fallot is the most common cardiac condition associated with brain abscess. Right-to-left shunt, hypoxia, acidosis, and increased viscosity decrease the perfusion in the brain resulting in micro infarcts, which provide the milieu for organisms to form abscesses. The 2 mortalities in this study were associated with cardiogenic brain abscesses.

Important criteria for evaluating treatment are the neurologic condition of the patient and abscess size on imaging. Cranial imaging should be performed immediately if there is clinical deterioration; after 1 to 2 weeks if there is no improvement; and on a biweekly basis for up to 3 months until clinical recovery is evident<sup>[11]</sup>. Culture positivity is low in cases of brain abscess. In the present study, organism growth is seen only in 19.6% cases. In a study done in India, only 20% culture growth was noted whereas in China only 13% showed organism growth<sup>[12]</sup>. Lack of anaerobic culture and use of antibiotics before samples are drawn may be the reasons behind the higher number of negative culture reports. However, metagenomics analysis and nucleotide sequence analysis are being used in some centers to identify the responsible organism and they have been able to identify bacteria that have never been incriminated as a cause for brain abscess<sup>[13]</sup>.

Tract hematoma, abscess cavity hematoma, extension of abscess into the ventricles, surgical site infection, meningitis, sinus thrombosis and mortality are known complications of brain abscess management. Apart from this, drug-related complications including minor rashes (potentially due to phenytoin use) are common side effects. We commonly use anti-epileptics at least for 1 month and continue if seizure is present. Cerebral hemisphere was the most common site (84.32%) of abscess in the present study, which was similar to the previous study done by Sharma<sup>[14]</sup> in the same institute. One patient developed hematoma adjacent to the aspiration site, which can be explained either by direct injury to the vessel during aspiration or reactive hemorrhage due to rapid decompression of the abscess. In the pre-CT era, mortality rate was about 40%-60%. This has decreased to 6%-17% currently<sup>[15,16]</sup>. Preoperative GCS remains the best prognostic factor<sup>[16]</sup>.

There were two deaths during this study (3.92%). The first mortality was a case of cyanotic congenital heart disease with brain abscess that died due to cardiac causes during hospital stay. The second mortality was also a child with large abscess with intraventricular rupture. Patients with intraventricular extension of abscess carry higher mortality rates of up to 48%, even in this modern era<sup>[17]</sup>.

In conclusion, with the advent of modern technology in radio imaging, mortality rates of brain abscess have improved over the decades as compared in the past. Outcomes may be dismal if not treated early which is particularly challenging given non-specific presentation of many patients with brain abscess. Thus, a high

index of suspicion is required in patients with features of raised ICP. Burrhole with aspiration is an excellent option for surgical management. Major craniotomy and excision should be preserved for multiloculated, recurrent, large size abscess cavities only. Culture positivity is very low, so longer broad spectrum intravenous and/or oral antibiotics help in early resolution. Involvement of the otorhinolaryngology team to address the primary source has further helped improve outcomes in cases of otogenic brain abscess.

## DECLARATIONS

### Authors' contributions

Collected all data: Kafle P, Sharma MR, Shilpakar SK, Sedain G

Followed all the patients and analyzed: Kafle P, Pradhanang A, Shrestha RK, Bhandari BR

Critical analysis, language editing and final approval: Groves C

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The data presented is original and obtained in our laboratory. It is available with the authors and can be made available if required.

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None.

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

All treatment and study were performed in compliance with our institutional standard and the Declaration of Helsinki and consent was taken for all patients before treatment.

### Consent for publication

Not applicable.

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Review

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# A concise review of immunotherapy for glioblastoma

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## Abstract

Glioblastoma (GB) is the most common and aggressive form of primary brain tumors in adults with a universally poor prognosis despite multimodal management including surgery, chemotherapy and radiation therapy. Among the novel therapeutic strategies, immunotherapy deserves particular attention with its potential to evoke biologic response and harness the host immune system. Considerable success achieved for other tumors has elicited great enthusiasm and prompted research on immunotherapy for GB. While the central nervous system has traditionally been thought of as an immune-privileged site, our understanding is being refined with emerging evidence. Several studies have been conducted and more are under way to establish the role of immunotherapy in management of GB. Immunotherapy of GB has yet resulted in mixed success with conflicting research findings, emphasizing the need for extensive study before its integration into routine clinical practice. Although there is a lot of room for improvement, immunotherapy for GB may be feasible and serve as a viable management strategy broadening and strengthening the therapeutic armamentarium to combat this deadly disease. Herein, we present a concise review of immunotherapy for GB.

**Keywords:** Glioblastoma, immunotherapy, glioma, vaccine, passive immunotherapy, active immunotherapy, cytokine therapy, central nervous system

## INTRODUCTION

Glioblastoma (GB) constitutes the most common and aggressive form of primary brain tumors in adults<sup>[1]</sup>. Management of newly diagnosed GB includes maximal tumor resection followed by adjuvant chemoradiotherapy. The landmark study by European Organisation for Research and Treatment of Cancer (EORTC)/National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) in 2005 has reported



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significant median overall survival (OS) benefit with addition of temozolomide (TMZ) to conventionally fractionated radiation therapy (RT), making adjuvant chemoradiotherapy followed by adjuvant TMZ the standard of care for newly diagnosed GB patients<sup>[2]</sup>. However, disease recurrence is exceedingly common despite multimodal management. Repeat surgery, systemic agents and RT may be used in the recurrent setting as salvage therapeutic options, nevertheless, the clinical course is typically progressive with almost all patients ultimately succumbing to their disease<sup>[3-5]</sup>.

Several treatment strategies are being explored to improve outcomes of patients with GB. Among these, immunotherapy deserves utmost attention with several studies assessing its role in GB management. Herein, we present a concise review of immunotherapy for GB.

## MAIN IMMUNOTHERAPY APPROACHES FOR GB

Given the infiltrating nature of GB, diffuse microscopic disease may be typically present beyond the tumor bulk at initial presentation. Thus, a successful therapy should specifically address the infiltrative tumor stem cells surviving after implemented treatments such as surgery, chemotherapy and RT. Immunotherapy may conceptually take part in achieving this task, on the premise that it may facilitate combating with resistant GB cells through boosting of the host immune system. While the central nervous system (CNS) has traditionally been thought of as an immune-privileged site due to prevention of cellular and molecular diffusion by the blood-brain barrier and absence of lymphatic drainage, our understanding is being refined with emerging evidence<sup>[6-8]</sup>. The presence of a functional CNS lymphatic system has been reported recently and enhanced the focus on immunotherapy for brain tumors<sup>[9]</sup>.

Herein, we review main strategies for GB immunotherapy.

### Cytokine therapy

The rationale of cytokine therapy is activation of the immune system through administration of immunomodulatory cytokines. Cytokines are secreted or membrane-bound proteins and potent immunomodulators with a critical role in immune system coordination<sup>[10-13]</sup>. Anti-tumor activities of cytokines have been reported in animal studies paving the way for consequent cytokine-based cancer treatment strategies. Among the multitude of cytokines including interleukins, interferons and hematopoietic growth factors, FDA approval is currently available for interferon alpha for adjuvant treatment of melanoma and for high-dose, bolus interleukin (IL)-2 for management of metastatic melanoma and renal cell cancer<sup>[13]</sup>.

An important issue in cytokine therapy is to achieve effective concentrations in the tumor without causing excessive toxicity. While local delivery by viral vectors has resulted in limited success for gliomas, intratumoral injection of IL-2 secreting allogeneic fibroblasts into GL261 tumors in mice has achieved increased survival<sup>[14-18]</sup>. Also, while IL-2 can induce over-differentiation of T cells and induce apoptosis of activated T cells, it may also activate CD4<sup>+</sup> FoxP3 TREG regulatory cells, thereby inhibiting T cell activation and tumor killing activity. In this context, IL-7, IL-15, and IL-21 may also be used.

Liposomes and biopolymer microspheres are alternative routes utilized for intratumoral delivery of cytokines. Also, cytokines have been used for delivery of toxins in an effort to combat glioma cells with considerable success<sup>[19-21]</sup>.

Utility of cytokine immunotherapy in combination with other therapeutic modalities is being investigated to exploit the synergistic activity against GB cells<sup>[22]</sup>. Briefly, cytokine immunotherapy has achieved encouraging results despite the need for further supporting robust evidence.



### Passive immunotherapy

Serotherapy and adoptive immunotherapy are passive immunotherapy strategies. Passive immunotherapy is based on delivering the patient immune cells or antibodies with the capability of targeting the tumor cells<sup>[23]</sup>. Unlike active immunotherapy in which the patient's immune system is boosted, passive immunotherapy doesn't include activation of host immunity. Infusion of LAK cells into the tumor bed was an initial attempt of passive immunotherapy in the earlier years<sup>[24,25]</sup>. Cytotoxic T lymphocytes (CTLs) were also studied for adoptive immune response<sup>[26]</sup>.

The use of monoclonal antibodies as a passive immunotherapy approach may result in killing of tumor cells through different mechanisms<sup>[27]</sup>. Sparing of normal brain tissue without tumor may be achieved if the antigen targeted by the monoclonal antibody is specifically expressed by the tumor only. Vascular endothelial growth factor (VEGF) is highly expressed in GB and targeted for therapeutic exploitation with bevacizumab (BEV). As a recombinant humanized monoclonal antibody, BEV is bound to VEGF-A and exerts antitumor effect. An improvement in progression free survival and maintenance in quality-of-life and performance status has been reported with addition of BEV to RT and temozolomide<sup>[28]</sup>.

Another potential target in GB is the epidermal growth factor receptor (EGFR)<sup>[29]</sup>. EGFR gene mutation typically in EGFR variant III (EGFRvIII) is very common in GB. In this context, the use of anti-EGFRvIII antibodies in combined modality GB management is an area of active investigation.

### Adoptive T-cell immunotherapy

Adoptive T-cell therapy offers an alternative immunotherapeutic approach. In this treatment, tumor-specific autologous T-cells undergo *in vitro* amplification and are consequently infused to the same individual for therapeutic exploitation. Advances in genetic engineering has paved the way for adoptive T-cell immunotherapy through generation of high avidity tumor-specific T-cells<sup>[30]</sup>. Chimeric antibody receptor (CAR)-based treatments and cytomegalovirus (CMV) adoptive T-cell immunotherapy have great potential for further therapeutic exploitation<sup>[31-35]</sup>.

A unique advantage of adoptive T-cell immunotherapy is the capability of expanding substantial amounts of tumor infiltrating T lymphocytes (TILs) *in vitro* without immunosuppressive environments seen *in vivo*<sup>[36]</sup>.

Adverse effects of adoptive T-cell immunotherapy may include cytokine release syndrome (CRS) and tumor lysis syndrome (TLS) which underscore the importance of early detection of these syndromes through vigilant monitoring<sup>[37,38]</sup>. CRS and neurotoxicity may be triggered by the inflammatory molecule IL-1, and adding ANAKINRA, an inhibitor of IL-1, to the treatment regimen can block the molecule. Also, inserting the IL-1 inhibitor gene directly into CAR-T cells may prevent CRS.

### Active immunotherapy (peptide vaccines, dendritic cell vaccines, heat shock protein vaccines)

Active immunotherapy is based on the premise that vaccination against tumor antigen stimulates an adaptive immune response against tumor cells. Target antigens include tumor-specific antigens (TSAs) expressed solely by the tumor and tumor-associated antigens (TAAs) expressed by both tumor cells and normal cells. While TSAs have a greater potential to evoke a more potent and specific immune response compared to TAAs, they are exceedingly rare. EGFRvIII, IDH-1/2 mutations (e.g., R132H), and CMV proteins are known TSAs expressed in GB and IL-13R $\alpha$ 2, HER-2, gp100, survivin, WT1, TRP2, EphA2, SOX2, SOX11, MAGE-A1, MAGE-A3, AIM2, SART1, and tenascin are TAAs expressed in GB<sup>[10]</sup>.

Heterogeneity of GBs warrants the demand for individualized, patient-specific and non-toxic immunotherapies. Attracted by the success of vaccination against hormone-resistant metastatic prostate cancer, researchers have focused on developing vaccines against GB<sup>[39]</sup>. Herein, we review peptide vaccines, dendritic cell (DC) vaccines, and heat shock protein (HSP) vaccines.

## Peptide vaccines

This strategy is a targeted approach including the direct administration of a selected protein or peptide antigen frequently used with an adjuvant such as keyhole limpet hemocyanin (KLH) to enhance the immunogenicity<sup>[10,40,41]</sup>.

In the context of peptide vaccines, most extensive study has focused on targeting of EGFRvIII<sup>[42-44]</sup>. EGFRvIII is a TSA which is solely expressed by GB cells and not expressed by normal tissues<sup>[45]</sup>. This kind of targeting single tumor specific antigens has the advantage of theoretically eliminating normal tissue toxicity. The mutant form of the EGFR gene containing an in-frame deletion of exons 2-7 is found in approximately 20%-30% of patients with GB and causes tumor cell proliferation<sup>[46-49]</sup>.

In the recent phase II randomized ReACT study assessing association of rindopepimut and BEV in EGFRvIII-positive relapsed GB patients reported the benefit of rindopepimut treatment with regard to multiple endpoints including the 2-year OS and PFS rates, and the authors concluded that rindopepimut administered with BEV induced a potent EGFRvIII-specific immune response leading to tumor regression and prolonged survival of recurrent GB patients<sup>[50]</sup>. Another phase II multicenter study of EGFRvIII peptide vaccination in newly diagnosed GB patients, Sampson *et al.*<sup>[45]</sup> reported significantly improved OS in vaccinated patients. A notable finding in this study was the loss of EGFRvIII antigen in most patients relapsing after vaccination, indicating a critical role for vaccine-induced immune response in tumor eradication. They concluded that a randomized phase III trial was needed to establish the role of EGFRvIII-targeted vaccination for management of GB patients<sup>[45]</sup>.

However, randomized phase III trial of rindopepimut for newly diagnosed EGFRvIII-positive GB patients failed to show an OS benefit in preplanned interim analysis, leading to early closure of the study<sup>[51]</sup>.

Overall, studies of Rindopepimut have demonstrated encouraging results worth further testing<sup>[52-54]</sup>. Nevertheless, GB TSAs with higher expression levels may achieve improved treatment results.

Other than EGFR related peptides, personalized peptide vaccination has been another area of investigation, resulting in encouraging therapeutic outcomes<sup>[55,56]</sup>. IDH-1 R132H mutation is a newer appealing TSA with a typically lower prevalence in primary GB compared to secondary GB and is being currently tested in clinical studies<sup>[57]</sup>.

## DC vaccines

DCs are efficacious antigen-presenting cells (APCs) capable of vigorously activating the T-cells to attain a durable immune response through slow processing of antigens<sup>[58-61]</sup>. These professional APCs have been judiciously utilized for GB management since they are appealing candidates for therapeutic exploitation. DCs can be categorized into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs)<sup>[62,63]</sup>. In the study by Dey *et al.*<sup>[63]</sup>, mice vaccinated with mDCs generated an improved antitumor T cell response compared to pDC vaccinated mice. The use of DC-based vaccines achieved impressive results for newly diagnosed GB patients<sup>[61]</sup>.

In the study by Prins *et al.*<sup>[64]</sup>, safety, feasibility, and immune responses were comparatively assessed for GB patients treated using DC pulsed with autologous tumor lysate or with synthetic glioma-associated antigens. The study revealed that DCs pulsed with autologous tumor lysates achieved improved anti-tumor immune response compared to DCs pulsed with synthetic glioma-associated antigens<sup>[64]</sup>. Nevertheless, DC vaccines may yet be skeptical in human clinical trials notwithstanding the promising results in animal models.

A recent study by Mitchell *et al.*<sup>[65]</sup> revealed that the efficacy of DC vaccination could be enhanced through pre-conditioning of the vaccination site with a recall antigen such as tetanus/diphtheria toxoid.

Approaches for modulation of DC migration may prove to be a viable treatment option, however, modification of autologous DCs is a difficult task with a high cost and workload.

Another appealing immunotherapy approach includes targeting of the glioma stem cells (GSCs) which are considered to take part in treatment resistance<sup>[30,66]</sup>. A survival benefit has been achieved in rodent GB models by use of GSC-antigens loaded DC vaccination<sup>[30,67,68]</sup>.

### Heat shock protein vaccines

Targeting of a single TSA or TAA with vaccines limit the potential antitumoral effect to the subgroup of GB patients expressing those TSAs and TAAs. Single-antigen vaccines also suffer from the heterogeneity of the GB cells expressing the antigen which may lead to their diminished activity and usefulness. In this context, an alternative strategy has been developed including vaccination with a heat shock protein (HSP) peptide complex in order to achieve targeting of multiple antigens<sup>[69]</sup>. The concentrations of HSPs may reach high levels in the presence of protein misfolding, unfolding, or aggregation and under stress-inducing environments as in GB<sup>[69-72]</sup>.

The use of HSP-peptide complex in management of recurrent GB patients has been tolerated well and conferred an improvement of survival through enhanced immune response<sup>[73]</sup>.

In a study by Crane *et al.*<sup>[74]</sup>, the use of peptides bound to a 96 kD chaperone protein (HSP-96) for immunization of recurrent GB patients resulted in a median survival of 47 weeks after surgery and vaccination, indicating the efficacy of this approach. Another phase II trial by Bloch *et al.*<sup>[75]</sup> reported the safety of HSPPC-96 vaccine in 41 recurrent GB patients and emphasized the need for vigilance for pretreatment lymphopenia as a factor impacting outcomes of immunotherapy.

The utility of another glioma-associated antigen HSP47 was suggested to induce CTL responses with the potential of therapeutic exploitation for GB patients<sup>[76,77]</sup>.

### Immune checkpoint therapy

Immune checkpoint inhibitors are immunomodulatory therapeutics with the capability of blocking inhibitory molecules and their receptors on effector immune cells with a resultant T-cell response against various cancers<sup>[78]</sup>. Immune checkpoint therapy offers a viable immunotherapy strategy targeting the regulatory pathways in T cells to evoke an immune response against the tumor<sup>[79]</sup>. Boosting of the antitumor immunity by immune checkpoint inhibitors mediating the T-cell response has been an appealing strategy for therapeutic exploitation<sup>[80,81]</sup>.

Among the multitude of immune checkpoint molecules under current investigation and development for GB, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death 1 (PD-1) are the most popular given the favorable outcomes achieved for other tumors through their inhibition, leading to FDA approval<sup>[82-86]</sup>. Studies have suggested the ability of these immune checkpoint inhibitors to overcome the blood-brain barrier for activity within the CNS<sup>[87-90]</sup>.

In the context of GB, their inhibition showed promise in preclinical trials<sup>[91-95]</sup>.

Although the first phase III study of PD pathway inhibition, the Checkmate 143 trial failed to meet its primary endpoint, several studies have focused on immune checkpoint inhibition for GB patients<sup>[96-98]</sup>.

As well as the enthusiasm for therapeutic exploitation of immune checkpoint blockade, there have also been important concerns about immune-related toxicity profile of immune checkpoint inhibitors, partly due to

increased amount of proinflammatory cytokines along with aberrant infiltration of stimulated T cells into normal tissues<sup>[99,100]</sup>. Nevertheless, there is a lot of room for further improvement and immune checkpoint blockade may serve as a viable immunotherapeutic strategy for patients with GB.

## **FUTURE DIRECTIONS**

Immunotherapy for GB is being thoroughly investigated in a plethora of studies to establish the safety and efficacy for therapeutic exploitation. Results of critical studies are eagerly awaited before decision making for integration of immunotherapy into clinical practice of GB management. A few points to be considered for GB immunotherapy are as follows:

- Further investigation and understanding of the immune system evasion mechanisms may assist in improved therapeutic exploitation of personalized immunotherapeutic strategies for GB patients. Focusing on molecular subtypes of GB and identification of molecular factors affecting the interplay between the tumor and immune system may be critical for developing personalized treatments for patients suffering from this deadly disease.
- Measurement of immune response may be further optimized through the introduction of standardized and validated assays, which play a central role in therapeutic decision making for a given immunotherapeutic.
- Given the grim prognosis of GB patients, current standard management may be judiciously supported by boosting of antitumor response with immunotherapy. In this context, achieving an improved therapeutic ratio for GB patients may warrant the utilization of combination therapies with incorporation of immunotherapeutic approaches to exploit the advantage of synergistic antitumor activity of multiple treatment modalities.

Clearly, well-designed clinical trials are needed to assess efficacy and safety of combined modality GB management using immunotherapeutic agents. Improved understanding of the interactions between chemotherapy, radiotherapy and immunotherapeutic strategies will shed light on further research for optimization of more potent treatment of GB patients.

## **CONCLUSION**

Recent years have witnessed unprecedented advances and breakthroughs in basic and translational cancer research, leading to significant improvements in therapeutic outcomes for several tumors. Utilization of immunotherapeutic strategies proved to be efficacious against several cancers, leading to their thorough investigation for management of GB patients. Immunotherapy of GB has yet resulted in mixed success with conflicting research findings, emphasizing the need for extensive study before its integration into routine clinical practice. Although there is a lot of room for improvement, immunotherapy for GB may be feasible and serve as a viable management strategy broadening and strengthening the therapeutic armamentarium to combat this deadly disease.

## **DECLARATIONS**

### **Authors' contributions**

Concept and design of study: Sager O, Dincoglan F, Dirican B, Beyzadeoglu M

Drafting the article: all authors

Revising the article critically for important intellectual content: Sager O, Dirican B, Beyzadeoglu M

Final approval of the version to be published: all authors

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Technical Note

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# Classification of brain tumor using devernay sub-pixel edge detection and k-nearest neighbours methodology

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## Abstract

Any disease can be treated only once it is imaged, detected and classified. This paper proposes a set of algorithms for classification of a brain tumor with better accuracy and efficiency. The proposal uses a JPEG format of the DICOM image fed into three stages namely pre-processing, segmentation using sub-pixel edge detection method and using the nearest neighbor methodology for the detection and differentiation of benign and malignant tumors.

**Keywords:** Brain tumor, magnetic resonance imaging, k-nearest neighbor, sub-pixel edge detection, contrast enhancement, malignant, benign, classification, medical image processing

## INTRODUCTION

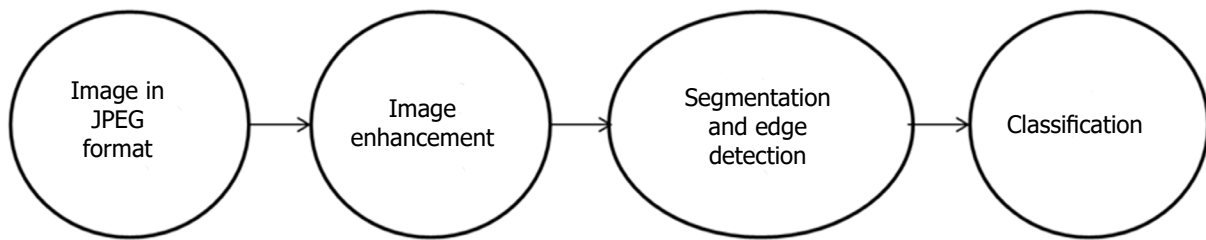
The field of biomedical imaging is highly used in today's life for detection of even the smallest possible abnormality in the human body. The primary goal of medical imaging is to extract the meaningful and accurate information according to the region of interest from the images with better accuracy and least possible error output. The various types of imaging methods in the scope of biomedical imaging are computerized tomography (CT) scans, X-rays and magnetic resonance imaging (MRI) scans<sup>[1]</sup>.

CT-scans make use of computer-processed combinations of many X-ray measurements taken from different angles to produce tomographic images of the region of interest of the scanned object, allowing the user to see



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**Figure 1.** The proposed system

inside the object without appending a cut on the human body<sup>[2]</sup>.

MRI scan works on the aspects of nuclei to produce detailed images of the human body. It uses a very powerful magnet to align the nuclei of atoms inside the human body and a variable magnetic field that causes the atoms to resonate. This phenomenon of resonance is called nuclear magnetic resonance. The atoms on resonating form a rotating magnetic field that the scanner detects and uses to create an image<sup>[3]</sup>.

Out of all the imaging techniques available for medical purposes, to create high resolution images, the best and non-invasive technique is MRI as it doesn't involve exposing the body to any kind of harmful radiations<sup>[2]</sup>.

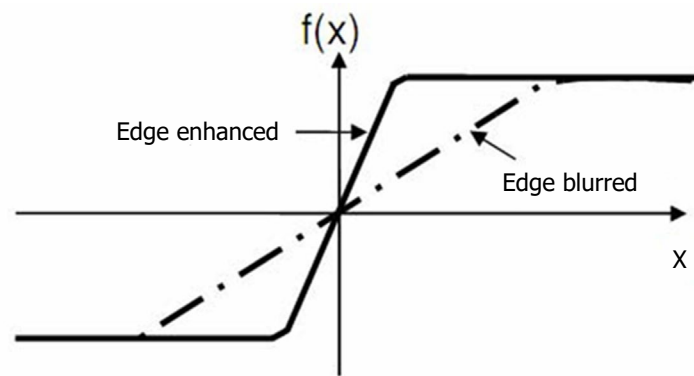
A tumor in any part of the body also known as a neoplasm is a mass formation due to the abnormal growth of the tissues. A tumor found inside the brain can be classified majorly as: primary - the tumor starts in the brain and tends to stay there; secondary - the tumor starts at any part of the body and traverse to the brain, in most cases cancerous in nature; malignant - the tumors which are deadly in nature and tend to increase in mass rapidly; and benign - the tumors which are non-cancerous in nature and generally do not cause any major loss to the human life<sup>[4]</sup>.

According to the American Brain Tumor Association, nearly 80,000 new cases of brain tumor are diagnosed every year within America and approximately 32% of these tumors are malignant or cancerous. Approximately 700,000 people in the USA are living with a tumor in the brain or the central nervous system. The trend also suggests that above 16,000 people will lose their lives to malignant tumors<sup>[5]</sup>.

Further adding on to the statistical trends in India approximately 10 people of every 100,000 people suffer from brain or central nervous system tumor. This is an increasing trend. Out of all the malignant tumor cases in India 2% cases are brain tumors<sup>[6]</sup>.

## PROPOSED SYSTEM

The main role of the system [Figure 1] as a whole will be to combine together various stages of detection and categorization of the tumor in the image. The first step of the system is the input system where an image of a brain tumor is given as an input. Image enhancement techniques have been widely used in many applications of image processing where the subjective quality of images is important for human interpretation. The next stage into the system is segmentation. Segmentation is the process of partitioning a digital image into multiple segments. The goal is to simplify and/or change the representation of an image into something that is more meaningful and easier to analyze. Image segmentation is typically used to locate objects and boundaries (lines, curves, *etc.*) in images. The resultant image of the segmentation process is the image showing the tumor highlighted in white. Then a feature matrix is calculated for the tumor and that is fed to a pre-trained classification algorithm which determines whether the tumor is malignant or benign. Executing this system as a whole is a challenging task for any person who is not a computer technician so the system



**Figure 2.** Image enhancement curve

proposes a graphical user interface (GUI) with interactive buttons. The use of GUI in such a system makes the task of executing the whole classification very easy for anyone. The user can easily segment the tumor and classify it to be benign or malignant in just clicks of buttons and within seconds.

An image from a MRI scanner is received in the form of a DICOM image. For better and faster computation, a DICOM image is converted into the JPEG format before being processed. Then the JPEG image is pre-processed mainly for the removal of noise and enhancement of the image quality. The processed image is then segmented using the sub-pixel edge detector method. Then the required computations are made to evaluate multiple values like mean, standard deviation, IDM, skewness, correlation, and homogeneity. Finally a k-nearest neighbor algorithm is applied for the classification of the tumor in accordance to the above computed values.

### Stage 1: enhancement of the tumor image

Image enhancement is used in medical imaging to make the images clearer and to ensure optimum presentation of all digital computer processing [Figure 2]. The importance of enhancement is to aid the interpretation by both humans and computers. Enhancement aims at improving the quality of image by removing noise, enhancing contrast, emphasizing edges and modifying shapes. Many computerized techniques are widely used and applied including the histogram equalization, linear shift invariant filters and morphological filters. For contrast enhancement there are two basic approaches, the first is Top-Hat, where the algorithm enhances the segmented edges of the region of interest and the second where the algorithm deals with the contrast of original image to enhance the segmentation process<sup>[7]</sup>.

### Stage 2: segmentation using sub-pixel edge detection technique

Sub-pixel edge detection consists of multiple stages within itself including edge detection, computing the gradient vector field, computing the sub-pixel edge points, chaining edge points and thresholds with hysteresis<sup>[5]</sup>. The main motive of the use of an edge detection algorithm is to segment out the edges of the tumor which is detected in the image. The sub-pixel edge detection works on the edges detected by the Canny or the Devernay algorithm for the refinement of the edges using the region-based segmentation techniques on the edges themselves. The line segments consisting of multiple pixels within the edges are basically modified to depict the edges using the minimum number of pixels<sup>[8]</sup>.

#### Algorithm 1: image\_gradient

Input - an image I, scale parameter S;

Output - the image gradient field vector g;

Step 1 - compute and derive the Gaussian filter of standard deviation S;



Step 2 - blur the image by convolution with the Gaussian filter derived in Step 1;

Step 3 - compute the x and y components of the image gradient by centered differences.

*Algorithm 2: compute\_edge\_points*

Input - the image gradient vector field g;

Output - a list of sub-pixel edge points;

Step 1 - if the gradient modulus of a given pixel is larger than gradient modulus of the left and right pixels then dub the horizontal edge point provided the gx component of the image gradient is larger or equal to the gy component;

Step 2 - analogously, a pixel whose gradient modulus is larger than the gradient at the pixels above and below is dubbed a vertical edge point if the gy component of the image gradient is larger or equal than the gx component;

Step 3 - if gradient modulus of a given pixel is larger than gradient modulus of the above and below pixels then dub the vertical edge point provided the gy component of the image gradient is larger or equal to the gx component;

Step 4 - finally the Devernay scheme is used either along the vertical or the horizontal axis to compute the sub-pixel position of the edge points.

*Algorithm 3: chain\_edge\_process*

Input - the image gradient vector field and a list of sub-pixel edge points computed above;

Output - modified edge list;

Step 1 - each point in the list is evaluated;

Step 2 - the neighbors of each pixel is computed;

Step 3 - edge points are associated with the pixel that is local maximum of the gradient modulus, either vertically or horizontally;

Step 4 - two subsets of the set are formed. The first set for forward chaining and the other for backward chaining;

Step 5 - the elements of each of these subsets with shortest distance of the edge point are selected as the candidates for forward and backward chaining;

Step 6 - then towards the end of the algorithm the previous chains are verified, both unlinking of previous chains and creating of new links are done if the new ones are better.

### Stage 3: classification using the k-nearest neighbors

K-nearest neighbor (k-NN) methods is a non-parametric regression methodology. An analytical function of the input-output relationship is used to K closest training vector for a given input feature vector. This algorithm function uses a distance (Euclidean) and a voting function. Other algorithms of the type k-NN also work in two phases of training and testing. In the training phase the data points are given to a n-dimensional space, n being a positive integer. In the testing phase the test data points are fed to the algorithm which in turn generates the nearest data points to the plotted data points on the n-dimensional plane<sup>[3,9-11]</sup>.

*Algorithm 4: k-NN classifier*

Step 1- determine a suitable distance matrix;

Step 2 - in training phase- plot all the data set pairs as points on a n-dimensional plane;

Step 3 - the data to be tested is plotted in the same plane and the nearest distance matrix is evaluated;

Step 4 - the k-NN are chosen and told to vote for the correctness of the algorithm to determine the best plausible classification result.

### Experimentation

A dataset of 210 images of brain tumors of various types was collected initially with the type of the tumor

**Table 1. Results and calculations from the testing phase**

Methodology	No. of excepted results	Accuracy percentage
Using DWT, PCA & k-NN	69/75	92%
Using contrast enhancement, sub-pixel edge detection and k-NN	71/75	94.67%

DWT: discrete wavelet transform; PCA: principal component analysis; k-NN: k-nearest neighbors

already known. As we know any classification algorithm needs two datasets: train data and test data. So a set of 80 data is initially selected for purpose of training the system to predict the results. The data or the feature matrix is created that stores the values and the labels for all the images in the train data. The rest of the 150 images are used for testing the system to calculate the accuracy percentage of the overall system.

## RESULTS

The system is run to distinguish between the two major types of brain tumors - malignant or benign for a dataset of 150 images. Images were equally distributed in the two types, with 75 each for benign and malignant. The figures below are screenshots of the GUI of the system as described above.

In the GUI, there are two buttons first for the load MRI image which is used to select the image of an affected brain from any folder within the computer or within any other connected device. Once the image is loaded into the system it is visible on the left axis and the system goes into standby mode till the user clicks the next button. Once the segment image button is pressed the segmented image is shown on the right axis. The same button also fills up all the edit text field values on the right end under the features tab. These values are calculated from the extracted features of the segmented portion of the image. These 13 values form up the feature matrix that is further used in the classification process. The text-field in front of the type of tumor tab gives the final result that is predicted by the k-NN classifier algorithm in the system.

**Figure 3** represent two different tumor images from the test data set that were run on the system and gave out the results as benign tumors. These classifications are completely based on the values of the various features that are calculated during the classification process.

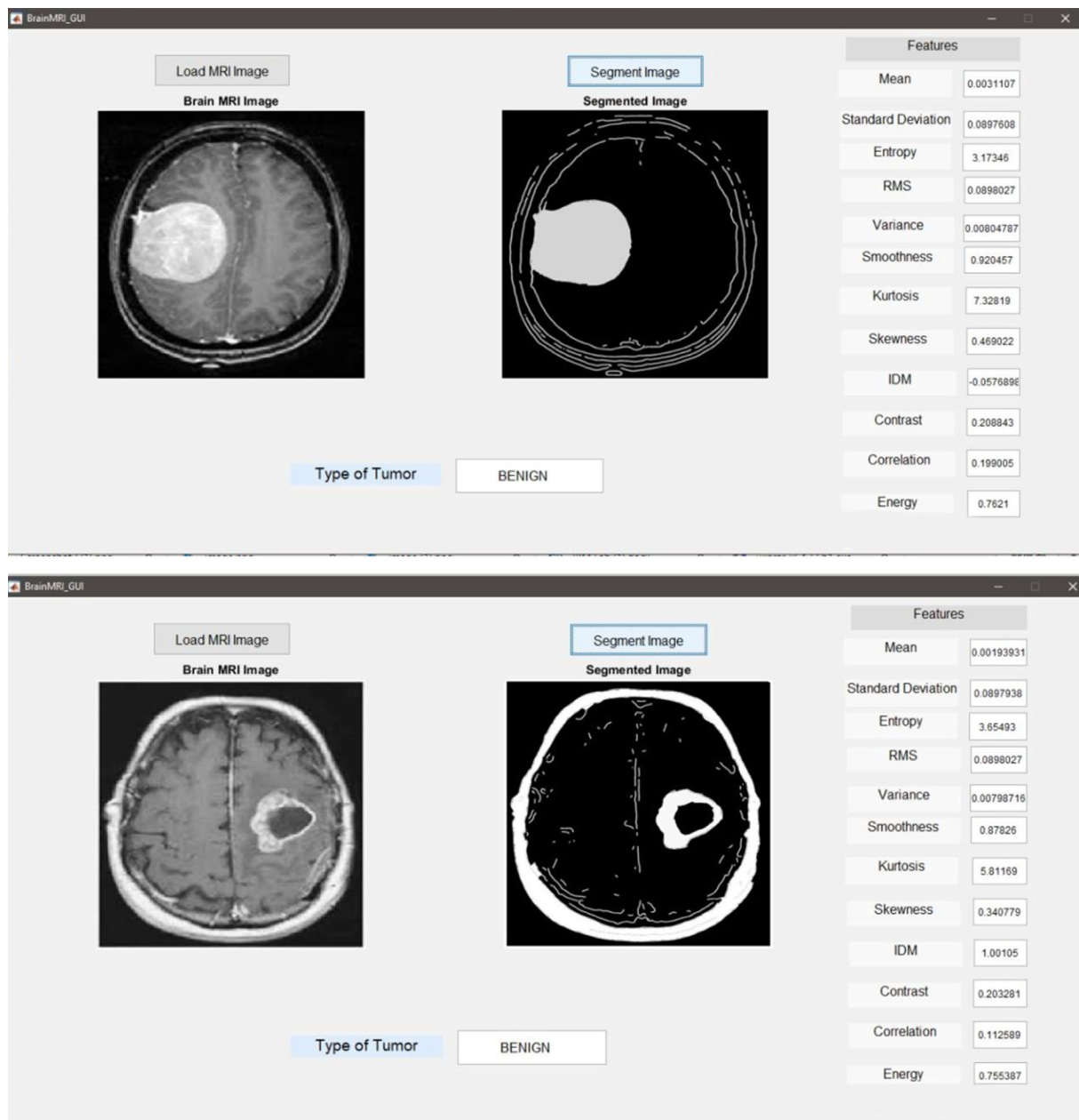
**Figure 4** represent two different tumor images from the test data set that were run on the system and gave out the results as malignant tumors. The system also effectively provides the segmentation and classification results for tumors of small sizes.

## ANALYSIS AND CONCLUSIONS

The feature matrices calculated in the above step are the main factors of the decision making process of the system. Here the 150 images within the test data set are considered wherein 75 images were benign and the other 75 were malignant. The system was then run for both sets of images - benign and malignant, and results were noted. The same process was run on for 100 iterations for the sole purpose of testing. Once the errors in each set were classified the average of both sets was calculated. Following that the error in the 100 iterations were taken and averaged. Finally the average value of error was taken and the accuracy of the system was calculated. Accuracy percentage = (correctness of the system/total number of observation)\*100%.

According to the data in **Table 1**, the average of 69 results were accurate out of 75 observations using the system that adopted discrete wavelet transform, principal component analysis and the k-NN algorithm for various stages like segmentation, feature extraction and reduction and classification process.

On the contrary, while using the latest methodologies such as contrast enhancement using the mathematical



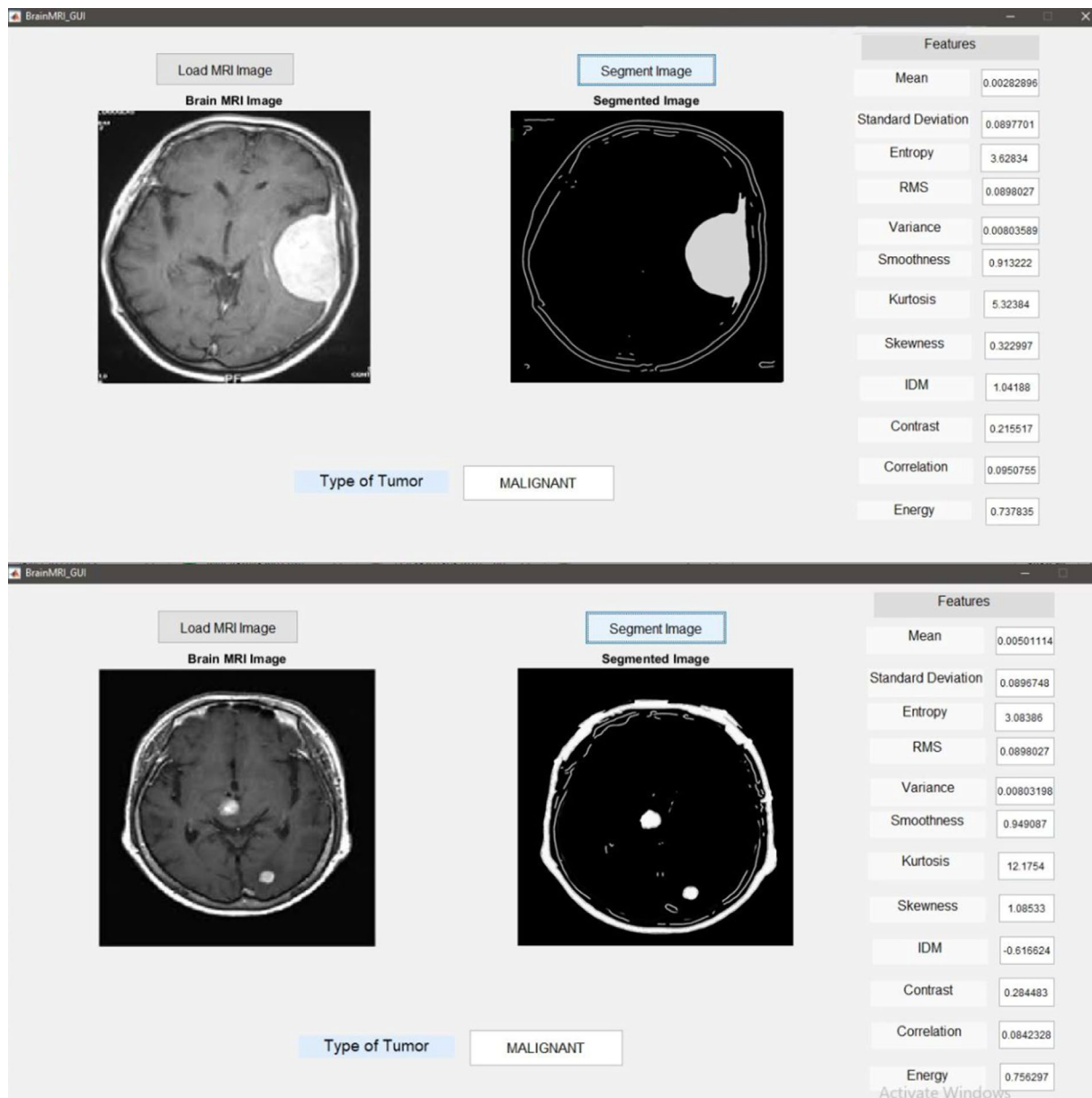
**Figure 3.** Results depicting a benign tumor

morphology, segmentation and feature extraction using the sub-pixel edge detection algorithm and the classification using the k-NN, a better observation of 71 accurate results in hundred iterations of testing was given. This leads to an accuracy percentage of about 94.67% on the selected data set.

The above results are capable enough to ensure the operation of the system as a whole on any given dataset.

## FUTURE ENHANCEMENTS

The speed of the system using DICOM images is still a challenge and we would want to focus on the DICOM images in the further enhancements. The other motive will be to collect more images for both train and test data sets and to have a better perspective on the testing of the system. The accuracy of the system can then only be confirmed with the highest level of accuracy.



**Figure 4.** Results depicting a malignant tumor

## DECLARATIONS

### Authors' contributions

Concept and design: Arora A

Data acquisition: Kumar R

Data analysis: Arora A, Tiwari S

Manuscript preparation: Arora A, Shwetha M

Critical revision and finalizing of the manuscript: Venkatesan S, Babu R

### Availability of data and materials

The data were strictly obtained from medical records according to the privacy policy and ethics code of our institute.

**Financial support and sponsorship**

None.

**Conflicts of interest**

All authors declared that there are no conflicts of interest.

**Ethical approval and consent to participate**

Due consent was taken from the patients to participate in the study and separately for the photography. Ethical approval was done according to the hospital and department policy.

**Consent for publication**

Not applicable.

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Review

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# Photodynamic therapy mediated immune therapy of brain tumors

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## ABSTRACT

Photodynamic therapy of tumors requires the topical, systemic or oral administration of a photosensitizing compound, illumination of the tumor area by light of a specific wavelength and the presence of oxygen. Light activation of the photosensitizer transfers energy to molecular oxygen creating singlet oxygen, a highly reactive and toxic species that rapidly reacts with cellular components causing oxidative damage, ultimately leading to cell death. Tumor destruction caused by photodynamic therapy is not only a result of direct tumor cell toxicity via the generation of reactive oxygen species but there is also an immunological and vascular component involved. The immune response to photodynamic therapy has been demonstrated to significantly enhance its efficacy. Depending on a number of factors, including type of photosensitizer, light dose and dose rate, photodynamic therapy has been shown to induce cell death via apoptosis, necrosis, autophagy and in particular immunogenic cell death. It is the purpose of this review to focus mainly on the role photodynamic therapy could play in the generation of specific anti-tumor immunity and vaccines for the treatment of brain tumors.

**Keywords:** Photodynamic therapy, photochemical internalization, photodynamic therapy induced cell death, anti-brain cancer vaccine

## INTRODUCTION

Tumor resection is the primary treatment employed in the treatment of high grade gliomas (HGG). The main functions of surgery are decompression of the brain, obtaining a histopathological and molecular



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classification of the tumor and reducing tumor load, allowing maximum effect of postoperative therapy. Despite employing modern imaging and surgical techniques, that increase the incidence of gross tumor resection, the tumor invariably recurs, usually in the vicinity of the surgical resection cavity<sup>[1,2]</sup>. The therapeutic goal following gross surgical resection of brain tumors therefore is to prevent recurrence by eliminating the infiltrating tumor cells both remaining in the margins of the resection cavity as well as remote from the tumor site. With this goal in mind anti-cancer immunotherapy is being actively researched as an important therapeutic modality for the treatment of HGG. Of the several methods to induce effective anti-tumor immune response, photodynamic therapy (PDT) has some potentially unique properties. PDT can induce combinations of apoptosis, autophagy and necrosis and immunogenic cell death, depending on a number of factors including type of photosensitizer, light dose and dose rate. It is the purpose of this review to focus mainly on the studies related to PDT-generated anti-tumor immunity and vaccines for gliomas.

### Photodynamic therapy

PDT of tumors requires the topical, systemic or oral administration of a photosensitizing compound, illumination of the tumor area by light of a specific wavelength and the presence of oxygen<sup>[3-8]</sup>. The photon energy activates the photosensitizer and initiates a complex photochemical reaction that generates cytotoxic reactive oxygen species (ROS) as shown in Figure 1. The light activated photosensitizer (PS) transfers energy to molecular oxygen creating singlet oxygen, a highly reactive and toxic species that rapidly reacts with cellular components causing oxidative damage, ultimately leading to cell death. Singlet oxygen causes mainly membrane damage by oxidizing amino acids, unsaturated fatty acids and cholesterol. Both the cell membrane as well as intracellular membranes such as mitochondria, endo-lysosome and endoplasmic reticulum damage is induced by PDT is largely dependent on the type of photosensitizer used<sup>[8]</sup>.

Different photosensitizers react with specific intracellular organelles, resulting in cell death via several varied mechanisms [Table 1].

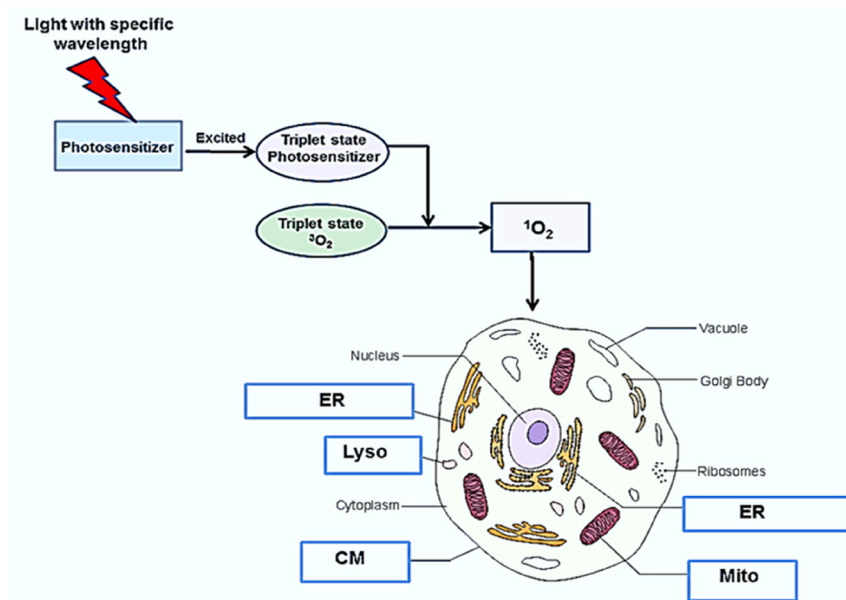
Unlike ionizing radiation and many chemotherapeutic agents, PDT does not exert its effects via DNA damage<sup>[7]</sup>. Additionally, PDT is a highly site-specific form of treatment, since its effect is restricted to the immediate vicinity of the region of illumination.

### PDT TREATMENT INDUCED ANTI-TUMOR IMMUNITY

Tumor destruction caused by PDT is not only a result of direct tumor cell toxicity via the generation of ROS but it is well established that there is also a significant immunological component involved. The great majorities of experimental studies have been done on extra-cranial tumors, and are reviewed in several extensive recent publications<sup>[9-14]</sup>.

The first evidence for induction of a tumor-specific immune response came by the demonstration that normal mice cured by PDT were able to resist a re-challenge with tumor cells in a tumor-specific manner<sup>[15]</sup> while immunosuppressed counterparts were not able to resist the re-challenge. Induction of systemic and memory immunity following PDT treatment has been verified in numerous studies. Systemic immunity following PDT treatment has been demonstrated by the ability of a locally induced immune response to affect distant non-treated areas<sup>[16-19]</sup>. Treatment of subcutaneous (s.c) primary tumors that led to 90%-100% of tumor ablation after PDT treatment showed a significant reduction of metastasized lung tumors compared to non-treated controls. In particular, a study using s.c colon carcinoma treated with hypericin-PDT yielded 100% of tumor cures, and i.v. re challenge with viable tumor cells showed no development of new tumors<sup>[20]</sup>.

PDT has been shown to induce apoptosis, necrosis, autophagy and immunogenic cell death (ICD)<sup>[21]</sup>. ICD is a cell death mode where the dead and dying cancer cells expose and/or release damage associated molecular



**Figure 1.** Mechanism and targets for photodynamic therapy. Following photosensitizer administration, light of a particular wavelength matching an absorption resonance of the photosensitizer, is used to excite the molecule up to a triple state. The excited photosensitizer transfers energy to ground state molecular oxygen ( $^3\text{O}_2$ ) resulting in the generation of singlet molecular oxygen ( $^1\text{O}_2$ ), a potent reactive oxygen species, resulting in cell death. cell membrane (CM) mitochondria (Mito), endosome, lysosome (Lyso), and endoplasmic reticulum (ER)

**Table 1. Typical photosensitizers and intracellular targets**

Photosensitizer	Intra-cellular organelle	Cell death mechanism	References
5-aminolaevulinic acid (5-ALA)	Mitochondria (Mito)	Apoptosis	[8,46,50,65]
Hematoporphyrin (HMME)	Cell membrane (CM)	necrosis	[7,8,51]
Hypericin (HYP)	Endoplasmic reticulum (ER)	ICD	[8,20,54,58]
Disulfonated aluminum phthalocyanine (AlPcS2a)	Endosomes, Lysosomes (Lyso)	Apoptosis, autophagy ICD?	[8,15,29,31,62]

ICD: immunogenic cell death

patterns (DAMPs). Although DAMPs are present in cells under normal conditions, they are exposed on the cell surface or released from cells upon the damage caused by the ROS generated by PDT. DAMPs reported to be necessary for the generation of antitumor immunity and induced upon PDT include surface calreticulin (CRT), heat shock protein (HSP) 70, HSP90, secreted adenosine triphosphate (ATP), and high-mobility group box 1 protein (HMGB1)<sup>[21-25]</sup>. Importantly, DAMPs cause maturation, activation and antigen processing/presentation of APCs, leading to their migration and proliferation in local lymph nodes. The mature APCs in the lymph nodes present the tumor antigens to a specific subset of CD8<sup>+</sup> T cells.

In addition, several studies have shown that PDT-treatment of extra cranial tumors followed by direct intra-tumoral injection of immature DCs, leads to an enhanced anti-tumor immune response compared to PDT treatment as single therapy<sup>[26-28]</sup>. This strategy induces *in situ* DC activation which enhances antigen acquisition and processing as well as migration of the DCs to draining lymph nodes.

### Photochemical internalization

Photochemical internalization (PCI), a derivative of PDT, has been shown to increase the efficacy of drugs, gene transfection as well as a variety of other anti-cancer agents that are taken up into cells by endocytosis<sup>[29-33]</sup>. PCI is based on the use of specially designed photosensitizers, such as AlPcS2a, TPPS2a, TPcS2a that localize preferentially in the membranes of endocytic and lysosomal intracellular vesicles.

Upon exposure to light of appropriate wave lengths, the photosensitizers induce the formation of short range singlet molecular oxygen, destroying the intracellular vesicles membranes, thus leading to the release of the contents of these vesicles into the cell cytosol. The released macromolecules can now exert their full biological activity instead of being degraded by lysosomal hydrolases.

Norum *et al.*<sup>[34]</sup> (2017) has examined the efficacy of PCI delivery of bleomycin (BLM-PCI) and its impact on systemic anti-tumor immunity in an extra-cranial mouse model. Their results showed that both PDT and BLM-PCI were incapable of inducing a curative effect in athymic mice at the light dose tested. In contrast, 50% of the light dose of that used in athymic mice resulted in a curative effect in 90% of the animals after BLM-PCI and 70% after PDT in normal mice. Inhibition of tumor cell growth was observed when combined with co-injection of splenic T cells from mice treated and cured with BLM-PCI. The anti-tumor immunity induced by BLM-PCI was equal to that obtained with PDT treatment, but at a lower light dose. Furthermore, the induced immune response after BLM-PCI was sufficient to reject tumor re-challenge immediately after PCI and lasted for at least two months.

An additional and novel method for enhancing the efficacy of peptide vaccines in extra cranial studies has been explored by Haug *et al.*<sup>[35]</sup> (2018) utilizing PCI to promote the escape of trapped endocytosed peptides into the cytosol of APCs. Their results showed that PCI caused a 30-fold increase in MHC class I/peptide complex formation and surface presentation on APCs, and a subsequent 30- to 100-fold more efficient activation of antigen-specific CTLs compared to using the peptide alone. These *in vitro* effects of PCI were translatable *in vivo* by the successful induction of antigen-specific CTL responses to cancer antigens in C57BL/6 mice following intradermal peptide vaccination and local light treatment. It is noteworthy that both macrophages and DC were used as APCs with approximately equal efficacy in these experiments. If these promising PCI strategies might be translatable to post-operative HGG treatment by the use of indwelling balloon light applicators, as has been proposed and tried for both radiation and PDT treatment, remains to be determined<sup>[36-39]</sup>.

### **PDT for the treatment of brain tumors**

PDT has been investigated as an adjuvant for the treatment of malignant gliomas for approximately 35 years<sup>[39-43]</sup>. Although numerous clinical trials have been initiated, the vast majority have consisted of uncontrolled phase I/II studies containing small numbers of patients. For example, in the single center phase III trial reported by Eljamel *et al.*<sup>[39]</sup> using both fluorescent guided resection combined with ALA and Photofrin repetitive PDT, a mean overall survival (OS) of the treatment group was 52.8 weeks compared to 24.6 weeks in the control group. In a phase II uncontrolled trial of 22 patients reported by Muragaki *et al.*<sup>[41]</sup>, using talaporfin sodium as a PS, a median of survival of 99 weeks was observed. This compared favorably to the 54-64 weeks OS obtained from previous trials employing standard post operative treatment consisting of radiation and TMZ. Due to differences in methodology and types of malignant brain tumors treated, it has been very difficult to evaluate PDT efficacy from these limited trials. For a more detailed account of the results of a number of PDT clinical trials for HGG, Bechet *et al.*<sup>[42]</sup> and Quirk *et al.*<sup>[43]</sup> give an excellent overview. Additionally, none of these clinical trials have included an evaluation of the effects of PDT on the immune response to treatment. Overall, the results of PDT trials for malignant gliomas have been relatively modest, thus providing the rationale for alternative PDT mediated treatment approaches such as PDT induced immunotherapy.

### **PDT mediated immunity of brain tumors**

There have been few experimental studies exploring the effects of direct PDT of brain tumors. Li *et al.*<sup>[44]</sup> showed that PDT *in vivo* generated regional and systemic anti-tumor immunity in mice with G422 gliomas in the brain. The infiltration of immune cells and the release of inflammatory factors, such as TNF- $\alpha$  and IFN- $\gamma$ , were increased in animals with G422 gliomas following PDT, compared to non-treated controls.

Splenic lymphocytes, isolated from PDT-treated mice, were able to induce anti-tumor immunity in nude mice. These workers could also demonstrate that PDT induced anti-glioma immunity was significantly reduced in tumor bearing complement C3 knockout as well as in nude mice.

Although PDT/PCI has clearly demonstrated the induction of a significant anti-tumor immune response, light based therapies are limited by the rapid absorption of light in tissue. For this reason the therapeutic efficacy of PDT, using presently available PSs, has been clinically confined mainly to superficial relatively flat tumors limited to skin and head and neck surfaces as well as bladder and esophagus. Effective PDT has been shown to extend only up to a depth of approximately 4 mm in cerebral tissue<sup>[45]</sup>. It would therefore not affect the glioma cells in more distant infiltration zones in the resection cavity wall, which can be measured in cm. In addition, the tumor cells infiltrating normal brain that lead to tumor recurrence are protected by the blood brain barrier, so uptake of PS can be inadequate<sup>[46]</sup>. To overcome the difficulties of *in situ* light delivery and dosimetry in postoperative brain tumor resection cavities, PDT produced anti glioma vaccines are a related approach that takes advantage of the immune stimulatory effects of *ex vivo* PDT of tumor cell cultures.

## PDT-PRODUCED CANCER VACCINES FOR GLIOMA

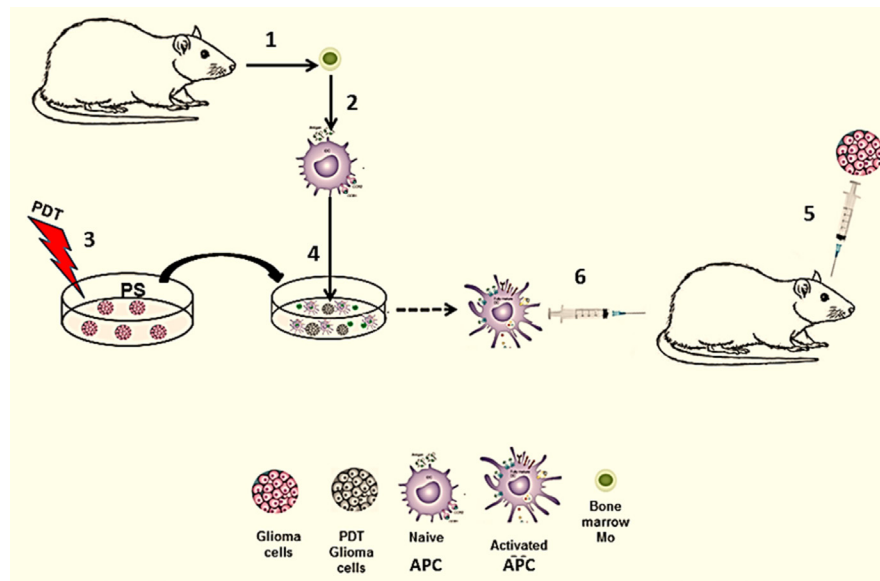
### *Ex vivo* produced vaccines

In earlier studies, using extra cranial tumor models, Gollnick *et al.*<sup>[47]</sup> demonstrated that lysates from PDT-treated tumor cells were more effective as preventative vaccines than tumor cells treated with UV, ionizing irradiation or cells subjected to freeze-thaw (F/T) cycles. Other groups have extended these results in several extra cranial models and could demonstrate that PDT-treated tumor cells could act as therapeutic anti-cancer vaccines<sup>[48,49]</sup>.

PDT generated vaccines against glioma cells have taken the form of CD activation by the use of crude tumor lysates, acid eluted crude lysates, and *in vitro* co-culture of PDT treated glioma cells and DC or macrophages (Ma) acting as APCs. Figure 2 illustrates the basic concept for an experimental PDT-APC co-culture vaccine.

The generation of vaccines in experimental models using PDT has been explored by a number of groups. For example, in an *in vitro* study, employing human glioma spheroids and dendritic cells from human donors, Etminan *et al.*<sup>[50]</sup> (2011) showed that ALA-PDT of glioma spheroids *in vitro* promoted DC attraction, uptake of tumor antigens and maturation of DCs, three important initial steps of the afferent phase of adaptive immunity. Co-cultured DCs with ALA-PDT-treated spheroids promoted the induction of CD83 (a marker for mature DCs) and upregulation of the co-stimulatory molecules CD40, CD80 and CD86. Additionally HSP-70 was upregulated on the spheroids after ALA-PDT treatment.

Shixiang *et al.*<sup>[51]</sup> generated DC vaccines using Photofrine-PDT-treated C6 glioma cell to produce antigenic peptides to activate DCs *ex vivo*. Immune response parameters between DC vaccines from PDT acid-eluted induced supernatants, DC vaccines from PDT-induced C6 supernatants or DCs exposed to antigens generated by direct acid elution only or freeze-thawing. Effects of these adaptively transfer DCs on host immunity were evaluated by measuring cytokine induction, as well as assessing DC-induced cytotoxic T lymphocyte (CTL)-mediated lysis of C6 target. Their results demonstrated that PDT-acid elution resulted in more effective DC differentiation associated with a high expression of CD80 and MHC-II compared with the other vaccine treatment groups. In addition the induction of the highest rat serum levels of IL-12 and TNF $\alpha$  and the lowest IL-10 levels were observed in the PDT acid eluted peptide group. Spleen cells isolated from these animals effectively mediated lysis of C6 target cells. They concluded that PDT of C6 cells significantly enhanced tumor cell immunogenicity compared to freeze-thawed C6 cells.



**Figure 2.** *Ex vivo* generated PDT-APC vaccine. (1) APC (DC/Ma) precursors obtained from donor animal; (2) cultured alone *in vitro* resulting in naïve APC; (3) *ex vivo* PDT treatment of tumor cells; (4) co-culture *in vitro* of treated tumor cells with naïve APC resulting in activated APC; (5) intra-cranial inoculation of glioma cells into the brain; (6) immunization with activated APC. APC: antigen presenting cell; PDT: photodynamic therapy; PS: photosensitizer

Reactive oxygen species (ROS) production and endoplasmic reticulum stress are believed to be important factors inducing ICD<sup>[52]</sup>. The photosensitizer Hypericin, a naturally occurring photosensitizer, mainly locates to the membranes of the endoplasmic reticulum and Golgi apparatus<sup>[53]</sup>. Hyp-PDT has been shown to induce major DAMPs characteristic of ICD including surface-exposed calreticulin (CRT), surface exposed HSP 70/90, secreted adenosine triphosphate (ATP), and passively released high-mobility group box 1 (HMGB1) protein<sup>[54-56]</sup>. ICD induced by Hyp-PDT was more effective in comparison to that induced by chemotherapy or radiotherapy<sup>[57]</sup>.

In a recent study Garg *et al.*<sup>[58]</sup>, combined HYP-PDT induced ICD with DC immunotherapy in an orthotopic HGG mouse model involving both prophylactic (immunization before i.c tumor cell implantation) as well as therapeutic (immunization after i.c tumor cell implantation) treatment protocols. Both protocols using ICD-based DC vaccines demonstrated a significant anti-HGG survival benefit. In particular using a therapeutic protocol, Hyp-PDT induced ICD-based DC vaccines together with chemotherapy (temozolomide) increased survival of HGG-bearing mice by up to 300%, resulting in half of the immunized animals becoming long-term survivors. Noteworthy was the observation that ALA-PDT treated glioma revealed no significant increase of CRT and release of HMGB1, two important DAMPs for the induction of ICD. The different characteristics of the various PSs used for PDT will in all probability determine their impact on subsequent antitumor immunity. Additionally, Hyp-PDT induced ICD-based DC vaccines appeared to induced an immune-stimulatory shift in the brain, from regulatory T cells to TH1/cytotoxic T lymphocyte/TH17 cells. A similar T cell shift has been shown to be associated with good patient prognosis in several tumor types<sup>[59,60]</sup>.

Although DCs have been used as APCs in the vast majority of immunization studies recent work has shown that DCs are part of the mononuclear phagocyte system and that they are indistinguishable from macrophages (Ma) in several key areas including developmental pathways, markers and efficacy as APCs<sup>[61]</sup>. Therefore, DCs it is argued, have no unique adaptation for antigen presentation that is not shared by other Ma and, as such, it is not surprising that both cell types are equally active vis a vis antigen presentation. We have used Ma together with the photosensitizer disulfonated aluminum phthalocyanine (AlPcS2a) mediated



PDT of F98 rat glioma cells *ex vivo*. ALPcS2a is a photosensitizer which enters the cell by endocytosis, and tends to localize in endosomes and lysosomes. PDT at relatively low light dose rates causes partial damage to lysosomes resulting in the release of hydrolases, which trigger both apoptotic and/or autophagy cell death.

Fischer rats and F98 (syngeneic) and BT<sub>4</sub>C (allogeneic) glioma cells were used in these experiments, in an *in vivo* brain tumor development model<sup>[62]</sup>. Co-incubation of naïve Ma with ALPcS2a-PDT treated F98 glioma cells led to pronounced morphological changes of the Ma. Naïve Ma were round in shape, approximately 10 to 15 µm in diameter and are composed of an equal population of both adherent and floating cells *in vitro*. By contrast, activated Ma were significantly larger, irregular in shape with increased intracellular inclusions and all of the cells were adherent in culture. Inoculation of these primed Ma (acting as APC), significantly inhibited but did not completely prevent the growth of F98-induced tumors in the brain. Complete suppression of tumor development though, was obtained via ALPcS2a-PDT-treated tumor cell primed Ma inoculation combined with direct intra-cranial injection of allogeneic glioma cells. Interestingly, allogeneic glioma cells injected into the brain in one hemisphere did not form tumors but surprisingly slowed the growth of syngeneic tumors induced in the contra-lateral hemisphere in the same animal. This appeared to indicate a systemic immune response generated via i.c inoculation by allogeneic glioma cells, though inadequate to prevent tumor development, did have an inhibiting effect.

Allogeneic cells likely contain antigen determinants shared with the syngeneic tumor, leading to the observed reduction in tumor growth. This hypothesis is in agreement with the previous findings of Stathopoulos *et al.*<sup>[63,64]</sup> in preclinical studies in rats using both allo and syngeneic stimulation. The underlying DAMPs developed by ALPcS2a-PDT, as has been previously demonstrated for Hyp-PDT<sup>[54-58]</sup>, remains to be determined in detail.

### ***In vivo* produced vaccines**

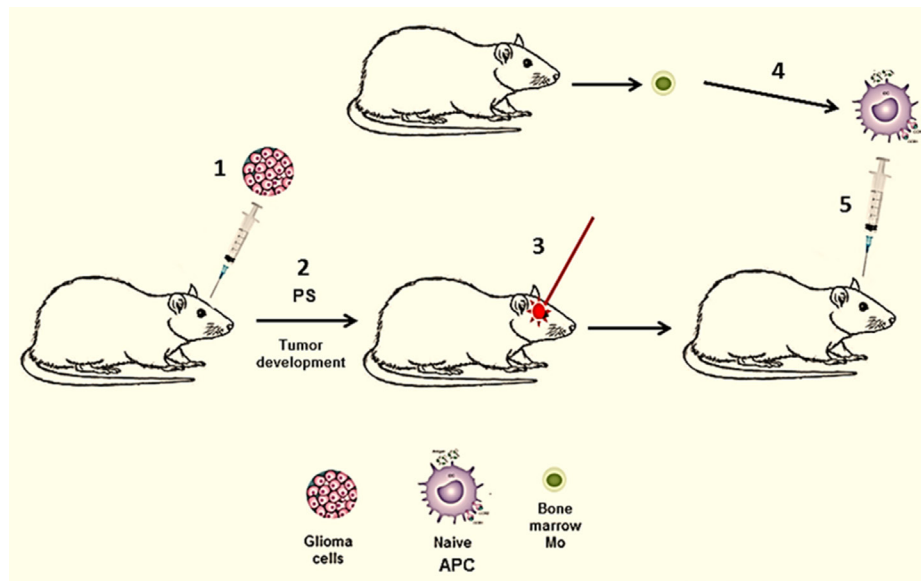
In all of the above mentioned studies glioma tumor cells were PDT treated *in vitro*. In a subset of brain tumor patients, harboring surgically inaccessible tumors, interstitial PDT (iPDT) has been evaluated<sup>[65]</sup>. Here light treatment is applied via stereotactically placed implantable fibers directly into the tumor. iPDT could be combined with direct injection of naïve APCs as has been done in several previously described extra-cranial experimental tumor models<sup>[26-28]</sup>. This protocol translated to intra-cranial tumors is illustrated in [Figure 3](#) and is presently under development.

This combined iPDT-APC injection strategy would both directly destroy portions of the tumor and additionally induce *in situ* APC activation which enhances antigen acquisition and processing as well as migration of the APCs to draining lymph nodes. This *in vivo* produced vaccine would potentially enhance the primary PDT effect and prevent tumor regrowth. It would also obviate the time consuming and costly necessity of priming APCs *in vitro*.

## **CONCLUSIONS**

Although the experience with PDT/PCI produced anti HGG vaccines is limited and no clinical trials have been done, PDT/PCI appears to be an inducer of immunogenic cancer cell death, an important step in the afferent phase of the immune anti-tumor response. Light activated induced immunotherapy therefore holds the potential to become a complementary therapeutic option for patients with HGG. Taking into account the penetration limitations of light activated therapies in the brain the further development of *ex vivo* PDT/PCI generated APC or peptide vaccines seems the most attractive approach. A deeper and detailed understanding of the induction of the antitumor immunity induced by light activated therapies would allow in the defining of protocols which would focus and enhance the immune system to recognize and prevent the inevitable post-operative recurrence of the tumor. Combining PDT induced anti-tumor vaccines with other therapeutic modalities including check-point inhibitors, is an exciting field to explore. Although





**Figure 3.** *In vivo* generated PDT-APC vaccine. (1) Intra-cranial inoculation of glioma cells; (2) tumor development, PS injection into the animal; (3) APC (DC/Ma) precursors obtained from donor animal, cultured alone *in vitro* resulting in naïve APC; (4) iPDT of tumor *in situ*; (5) immunization with naïve APC injection directly into PDT treated tumor. APC: antigen presenting cell; PDT: photodynamic therapy; PS: photosensitizer

not discussed in this review both PDT and PCI have an effect on the vasculature and have been shown to temporarily open the blood brain barrier in a limited site specific region<sup>[66-68]</sup>. What additional role this would play in the development of an effective and safe anti-HGG patient therapy, remains to be established.

## DECLARATIONS

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### Authors' contributions

Contributed to the overall preparation of the manuscript: Hirschberg H

Contributed the section on photochemical internalization and to revised drafts: Berg K

Contributed the section on photosensitizers and targets and to revised drafts: Peng Q

### Availability of data and materials

Not applicable.

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### Conflict of interest

All authors declare that they have no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Case Report

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# *Streptococcus gallolyticus* meningococcalitis in adults: the first case report in China

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## ABSTRACT

We report on the first case of a *Streptococcus gallolyticus* meningococcalitis in China. The bacterium was first isolated from the patient's cerebrospinal fluid and has so far not been associated with human infections of the central nervous system. We hope our case report can give some references for the diagnosis and treatment of the *Streptococcus gallolyticus* meningococcalitis in China.

**Keywords:** *Streptococcus gallolyticus*, meningococcalitis, China, case report, adult

## INTRODUCTION

*Streptococcus gallolyticus* is an uncommon cause of meningococcalitis so far apart from the bacteremia or the peritonitis. We report one case with meningococcalitis in which *Streptococcus gallolyticus* was isolated from the cerebrospinal fluid (CSF). The organisms were seen on a gram-stained preparation of CSF. The patient had a history of adenocarcinoma of colon and presented with hyperpyrexia, delirium and neck rigidity. After the antibiotic treatment for 2 weeks, finally he got fully recovery. A review of the literature revealed only about 42 cases of meningitis due to *Streptococcus gallolyticus* were reported and the present case report was the first one from China. The case indicates the importance of laboratory identification of specific organisms and provides experience in meningococcalitis caused by *Streptococcus gallolyticus*.



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**Table 1. Clinical characteristics for the patient with *Streptococcus gallolyticus* meningocephalitis**

Characteristics	Patient data
Age (years)	80
Gender	Male
Predisposing factor (s)	Adenocarcinoma of colon Bibulosity
Clinical presentation	
Temperature (°C)	39.3
Neck rigidity	Yes
Headache	No
Score on Glasgow Coma Scale	13
Neurological deficits	Delirium
CSF findings	
Leukocyte count	$74 \times 10^6/L$
Protein level (mg/L)	6058
CSF/blood glucose ratio	< 0.13
Cranial CT (MRI)	Normal
Cultures	
Blood culture	Negative
CSF culture	Positive
<i>S. gallolyticus</i> -associated disease	
Endocarditis	No
Colon adenocarcinoma	Yes
Strongyloidiasis	Unknown
Empirical treatment	
Antibiotics	Meropenem, linezolid
Dexamethasone	No
Outcome	Recovery

CSF: cerebrospinal fluid; CT: computed tomography; MRI: magnetic resonance imaging

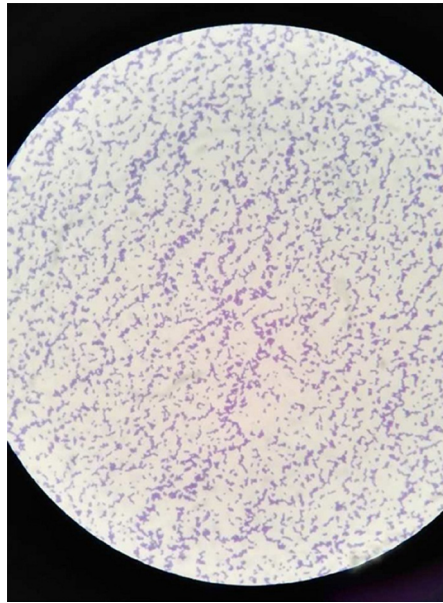
## CASE REPORT

An 80-year-old man was admitted to the emergency room of Guangdong Provincial Hospital of Chinese Medicine. He presented symptoms with deliration and neck rigidity. According to his wife's statement, the patient complained the abdominal pain 5 days ago. Meanwhile, he had a history of radical operation for adenocarcinoma of stomach and colon, as well as the percutaneous coronary intervention. Further questioning for his wife and daughter revealed that the patient has the habit of drink Chinese liquor for about 50 mL every day for decades before the partial resection of stomach. Even in the latest years, he still drank about 20 mL every day. The features of the patient are summarized in [Table 1](#).

On examination, he was delirious with neck rigidity, but no febrile. There was no other neurological abnormalities. Meanwhile, there is no abdominal pain. Peripheral blood samples, including cultures, were taken and treatment with ceftriaxone (2 g intravenous drip, ivd q12h) as well as acyclovir (75 mg peros, po bid) were started immediately. Urgent brain computed tomography (CT) scanning was normal. Then the lumbar puncture was performed. Laboratory studies of CSF disclosed the following values: the CSF pressure, 130 cmH<sub>2</sub>O; WBCs,  $15 \times 10^6/L$ ; glucose < 1.11 mmol/L (serum glucose: 8.96 mmol/L); and protein, 4946 mg/L. He was diagnosed with central nervous system (CNS) infection and was transferred to the neurological department.

Day 1 physical examination showed a poor-nourished man in a mild altered consciousness. His vitals were: temperature 36.4 °C, blood pressure 145/59 mmHg, pulse rate 66 and respiration rate 20. Neurological examination revealed a delirious man who had decreased range of motion of his neck. No other abnormalities of neurologic system as well as the respiratory and digestive system were detected. Then, blood routine, high-sensitivity C-reactive protein (hs-CRP), procalcitonin including cultures were taken again. Magnetic resonance imaging (MRI), electroencephalogram (EEG) monitoring was performed. Combining





**Figure 1.** The microscopic view of cerebrospinal fluid culture (1:1000)



**Figure 2.** The macroscopic view of *Streptococcus gallolyticus* (1:1)

the patient's symptoms, positive signs, results of the blood test and CSF test, we considered the patient suffered from meningocephalitis and the treatment with meropenem (2 g ivd q8h) was started. Meantime, a lumbar puncture was performed for the second time.

Blood routine showed the following values: WBCs,  $12.1 \times 10^9/L$  (87.9% neutrophils, 7.7% lymphocytes, 4% monocytes). And CSF test found the following values: CSF pressure, 110 cmH<sub>2</sub>O; WBCs,  $74 \times 10^6/L$  (15% neutrophils, 80% lymphocytes, 5% monocytes); glucose < 1.11 mmol/L (serum glucose: 8.33 mmol/L); and protein, 6058 mg/L. At this time, *Streptococcus gallolyticus* was isolated from CSF cultures [Figures 1 and 2]. Considering the permeability of linezolid through the blood brain barrier (BBB) and the sensitivity of linezolid (minimum inhibitory concentration, MIC /kindy-bauer, KB 29 mm) to the *Streptococcus gallolyticus*, the therapy with linezolid (0.6 g ivd q12h) was started.

Brain contrast enhancement MRI showed no signs of infection and the long-term EEG monitoring found no epileptic signs. The detailed report showed the background activity was no alpha wave and diffused slow

**Table 2. Comparison of the clinical characteristic and test results with the patient**

Characteristics	Day1	Day 3	Day 8	Day 14	Day 28
Clinical presentation					
Consciousness	Delirium	Consciousness	Consciousness	Consciousness	Consciousness
Neck rigidity	Yes	Yes	No	No	No
Headache	No	No	No	No	No
Fever	No	Yes	Yes	Yes	Unknown
Score on Glasgow Coma Scale	13	13	15	15	15
Temperature (°C)	36.4	37.8	38.3	38.2	Unknown
CSF findings					
Leukocyte ( $\times 10^6/L$ )	74	640	384	Unknown	32
Protein level (mg/L)	6058	5160	2342	Unknown	359
CSF glucose (mmol/L)	< 1.11	1.47	1.81	Unknown	Unknown
Blood findings					
Leukocyte ( $\times 10^9/L$ )	12.1	19.16	21.89	11.25	11.16
Neutrophils (%)	87.9	81.2	87.2	75	79
Monocytes (%)	4	6.7	4.8	10.8	7.1
Lymphomonocyte (%)	7.7	12	6.7	11.9	11.9
Serum glucose (mmol/L)	8.33	10.95	6.83	6.61	Unknown
Cultures					
Blood culture	Negative	Negative	Negative	Negative	Unknown
CSF culture	Positive	Negative	Negative	Negative	Negative
hs-CRP (mg/L)	Unknown	Unknown	57.04	34.03	25.3 (CRP)
Procalcitonin (ng/mL)	Unknown	0.58	0.17	0.17	0.04

CSF: cerebrospinal fluid; hs-CRP: high-sensitivity C-reactive protein

waves as well as low amplitude of beta were observed in all leads. No sleep cycle wave was observed as well.

Day 3, he developed a mild fever with the temperature of 37.8 °C. He became conscious but still cannot communicate with the others. A lumbar puncture was performed to evaluate the effect of the therapy. The WBCs in blood and CSF increased while the glucose and protein levels were improved [Table 2]. The cultures of CSF and blood were negative.

Day 8, the physical examination showed the neck rigidity was better than before and there were few rales in both lower lungs. Deep sputum cultures yielded multidrug-resistant *baumani* and the amikacin (0.2 g ivd q12h) was added.

As the neurological function was improved, we performed another lumbar puncture and the results showed the WBCs decreased to  $384 \times 10^6/L$  and protein as well as the glucose increased to 2342 mg/L and 1.81 mmol/L separately indicating the antibiotic therapy was effective. Controversially, the WBCs and hs-CRP in the blood increased. Considering the symptoms and the results of CSF tests were both improved, the therapy with meropenem and linezolid was continued.

While the treatment was effective based on the CSF results. We did the other tests to rule out the infection of the other systems and the cacotrophy conditions. An echocardiogram revealed no evidence of endocarditis and colonoscopy revealed the ascending colon polyps and haemorrhoids. The chest X-ray found no tumor. The abdominal CT scan was also negative.

The results of the marker for the systemic inflammation, CSF, clinical presentation are summarized in Table 2. All together, meropenem (2 g ivd q8h) was given for 16 days, linezolid (0.6 g ivd q12h) was given for 15 days and amikacin (0.2 g ivd q12h) was given for 5 days. Other treatments including nutritional supporting, early rehabilitation, bronchofiberscope, bed sore prevention were also given to the patients. Finally, the patient was completely awake and transferred to the rehabilitation facility.

Two weeks after discharge, we followed up the patient by telephone. His wife told us the lumbar puncture was performed once more in the rehabilitation facility and he could take care of himself independently. The laboratory studies of CSF and blood was almost normal [Table 2].

## DISCUSSION

There are only about 42 adult cases about *Streptococcus gallolyticus* meningitis reported according to the literature. For now, the present case report was the first from the Chinese population. The detail of the diagnosis and treatment was documented which could provide experience for the subsequent patients of the CNS infection with *Streptococcus gallolyticus*.

We think that the gastrointestinal tract was probably the source of *Streptococcus gallolyticus* in our patient who had prior gastroenteritis and had the history of moderately differentiated adenocarcinoma of stomach treated with partial resection together with colonic adenocarcinoma. Many studies have confirmed the fact that patients with both benign and malignant of gastrointestinal lesions were susceptible to *Streptococcus bovis* (*S. bovis*) bacteremia<sup>[1-3]</sup>. A review of 119 cases of *S. bovis* endocarditis or bacteremia suggested that there were 48 patients accompanied with gastrointestinal neoplasms, 22 of which were adenocarcinoma<sup>[4]</sup>. Colonic carcinoma has been reported in up to 50% of patients with *S. bovis* bacteremia or endocarditis<sup>[5]</sup>. So, there is a strong link between *Streptococcus gallolyticus* infection and bowel disease. Nevertheless, the extent, nature, and basis of this association are still not completely understood.

According to the literature, the susceptible risk factor for the *Streptococcus gallolyticus* meningitis was cacotrophy, immunosuppression, endocarditis, colon carcinoma, strongyloidiasis and bibulosity<sup>[6-10]</sup>. Among the 42 adult patients reported<sup>[11]</sup>, 43% (18/42) of the patients had the conditions such as immunosuppression, cancer and alcoholism. Meantime, 33% (14/42) of the patients had the infection with the strongyloidiasis, 63% (15/24) had the colon abnormalities, 8% (5/28) had the endocarditis. Furthermore, endocarditis has been reported to be caused by *S. gallolyticus ssp. gallolyticus* more frequently than by *S. gallolyticus ssp. Pasteurianus*<sup>[12,13]</sup>. So, it is necessary to test for strongyloidiasis and do the echocardiography and colonoscopy for the patients with *Streptococcus gallolyticus* meningitis. For our patient, it is definitely that he has the cacotrophy, colon carcinoma, but not the endocarditis. Unfortunately, in our patient, the stool examination was not performed repeatedly to detect strongyloidiasis.

Even though, the CNS infection has clinical characteristics, such as ardent fever, headache and neck rigidity. In many patients with CNS infection due to *Streptococcus gallolyticus*, the presenting differentiated from each other, as occurred in our patient. It was not until days after admission that our patient developed the febrile signs. The delayed fever may be the reason of his advanced age, the condition of cacotrophy or the nosocomial infection.

As we all know, bacterial infection of the CNS has the following characteristics of laboratory examination: the CSF pressure, WBCs, NEUT% and the protein were high, while the glucose in CSF was reduced. In our patient, we performed lumbar puncture repeatedly, but the CSF pressure was never higher than normal. This may be due to differences in individual immune responses. IgG and IgA but no IgM are seen in normal CSF because IgM has a larger molecular weight. Humoral immune responses often form antigen-antibody complexes; this reaction is often carried out in blood vessels, leading to severe vasculitis reactions in or near nerve tissue. It may be the characteristic for *Streptococcus gallolyticus* meningocephalitis which needs more clinical data. Meanwhile, we cannot ignore the fact that the cultures plays an important role for the differentiation of CNS infections. For the 42 patients, the positive incidence was 88% for CSF cultures and 87% for blood cultures<sup>[11]</sup>. We performed the blood culture repeatedly, the results were all negative. So we think that our patient might not be infected through blood but through the gastrointestinal tract.

Now, *Streptococcus gallolyticus* performed as a member of *S. bovis*, which has three hypotypes. Before then, *S. bovis* strains were divided into biotypes based on their ability to decompose the mannitol (biotype I) or not (biotype II)<sup>[14]</sup>. Biotype II was further subdivided into biotypes II.1 and II.2. Depending on the ability to produce acid from trehalose, to exhibit frequently b-glucuronidase, to degrade starch and b-galactosidase activity, Biotype II. 2 strains are distinguished from biotype II.1 strains. In 1990, Osawa<sup>[15]</sup> suggested a new species, *S. gallolyticus*, isolated from fecal excretion of a koala, for those organisms able to decarboxylate gallic acid. Subsequently, the further studies suggested that the *S. gallolyticus* species comprised *S. bovis* biotypes I and II/2<sup>[16]</sup>. Later studies about the sequencing of soda and DNA-DNA hybridization confirmed the need for the taxonomic change<sup>[17,18]</sup>. Therefore, Abdulmir et al.<sup>[6]</sup> suggested that the *S. gallolyticus* species includes three subspecies: *S. gallolyticus subsp. gallolyticus*, *S. gallolyticus subsp. pasteurianus*, and *S. gallolyticus subsp. macedonicu*. Among the three biotypes of *Streptococcus gallolyticus*, we know that *S. gallolyticus subsp. pasteurianus* causes meningitis, bacteremia, peritonitis, and chorioamnionitis in adults<sup>[19-21]</sup>. Since this was the first case of *Streptococcus gallolyticus* among Chinese population, we didn't detect the subtype because the technical reasons. We hope that our case report can provide some information for the detection of the *Streptococcus gallolyticus* related diseases and to make more precision diagnosis by the subtype detection.

From the review of the *Streptococcus bovis* infection of the CNS, we know that most cases of *S. bovis* infection can be treated with penicillin alone<sup>[22]</sup>. But as is known to all, the cultures plays an important role in the course of treatment. Savitch et al.<sup>[23]</sup> found that patients with *S. bovis* endocarditis were resistant to penicillin G. Several researches proposed that the empirical antibiotics should be chosen based on patient history, results of CSF gram stain and local community antibiotic resistance patterns<sup>[24]</sup>. For our patient, considering the history of the patient as well as the permeability of BBB to antibiotics, we chosen the meropenem (2 g ivd q8h) firstly<sup>[25]</sup>. We added linezolid as soon as the *Streptococcus gallolyticus* reported. Considering the results of sputum Gram stain we continued the treatment with meropenem. Eventually, our patient recovered very well as we follow up by telephone two weeks after the discharge.

The studies suggested that the mortality rate of the *Streptococcus gallolyticus* meningitis patients is about 24%<sup>[11]</sup>, but we cannot ignore the fact that the total number of reported patients with *Streptococcus gallolyticus* meningitis was small. So, it is of great significance to form a standardized and effective diagnosis and treatment program for *Streptococcus gallolyticus* meningitis.

For now, all of the reports are from European and American countries. There are no reports from China. We hope our case report can give some references for the diagnosis and treatment of the *Streptococcus gallolyticus* meningocephalitis in China.

## DECLARATIONS

### Authors' contributions

Drafting the manuscript, and literature review: Dai YM, Zhao M, Lu HJ

Revising the manuscript: Wang LX

### Availability of data and materials

The data and material used in the study could be open upon request.

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### Conflicts of interest

All authors declared that there are no conflicts of interest relevant to this article.

## Ethical approval and consent to participate

The study is approved and the patient consent is obtained.

## Consent for publication

Not applicable.

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Review

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# Immunotherapy for pediatric brain tumors

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## ABSTRACT

Immunotherapy, while effective against lymphoid cancers and some solid tumors, has shown less benefit against pediatric brain tumors. Tumor heterogeneity, a suppressive immune microenvironment, and the blood-brain barrier have the potential to diminish any immune-based approach and limit efficacy. More importantly, most pediatric brain tumors are immunologically quiescent, stemming from a low mutational burden. This review focuses on innate vs. adaptive immunotherapeutic approaches and describes how the immunologic context of pediatric brain tumors can help identify well-suited immunotherapies for our patients. In this framework, we will discuss past and current approaches using virotherapy, immunoconjugates, monoclonal antibodies, active immunization, and adoptive cellular therapy, and share our thoughts on how immunotherapy can cure children with brain tumors.

**Keywords:** Immunotherapy, brain tumor, pediatrics, virotherapy, active immunization, adoptive cellular therapy

## INTRODUCTION

The incidence of pediatric brain tumors varies by country and ranges between 1-5 cases/100,000 persons, with about 4600 primary central nervous system tumors diagnosed in the United States annually<sup>[1,2]</sup>. There are over 100 histologic subtypes of brain tumors, but the most common diagnoses in children are low-grade gliomas, particularly pilocytic astrocytoma (incidence roughly 0.8/100,000) and medulloblastoma (incidence roughly 0.4/100,000)<sup>[2]</sup>. Outcomes for recurrent malignant brain tumors in children remain poor, and brain tumors are the leading cause of cancer death in children<sup>[3]</sup>. Even when effective, surgery, radiation, and chemotherapy cause neurologic and neurocognitive morbidity. Many children with brain tumors who survive



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their disease have significant cognitive disability that limits their ability to live independently, progress fully in their education, or pursue a vocation<sup>[4]</sup>.

Immunotherapy attempts to leverage the high specificity of the immune system to target and eliminate cancer cells while leaving healthy cells undamaged. Chimeric antigen receptor (CAR) T cells and PD-1/PD-L1 monoclonal antibodies are the most impactful immunotherapies to date and have cured patients who otherwise had no curative option. Unfortunately, these successes have not significantly improved outcomes for most children with brain tumors. Understanding the immune environment in which pediatric brain tumors exist is requisite for identifying effective immune-based therapies for these diseases.

Historically, the blood-brain barrier and perceived sensitivity of deep midline structures to manipulation have limited investigators' ability to develop and deliver therapies for children with brain tumors. In the modern era, direct delivery methods, improved drug design, and surgical intervention involving brainstem and deep midline tumors drive the field forward. This review will indicate the routes whereby immunotherapies are delivered and mechanisms through which they selectively target the tumor, but will not unduly focus on the blood-brain barrier or tumor delivery methods.

### IMMUNOLOGICALLY “HOT” VS. “COLD” TUMORS AND MUTATIONAL LOAD

“Hot” vs. “cold” tumors are distinguished by whether significant numbers of tumor infiltrating lymphocytes (TILs), most notably T cells, are present<sup>[5]</sup>. Functionally, T cells are potently cytolytic and are important for immunologic memory and surveillance to maintain an anti-tumor immune response<sup>[6]</sup>. T cell homing is influenced by activating cytokines, the tumor vasculature, integrins, and the presence of tumor-specific proteins, called “neoantigens”<sup>[5]</sup>. Hot tumors supply inflammatory cytokines and allow T cells permissive access within the tumor bed. Cold tumors lack T cell infiltration either because of a harshly immunosuppressive tumor microenvironment, or because the tumor is not inflammatory or exists in a strictly immune-privileged site. Whereas the central nervous system (CNS) was formerly regarded as immune-privileged, this notion has been dispelled; immune cells are highly adept at reaching the CNS, even without blood brain barrier disruption<sup>[7]</sup>.

In addition to inflammatory cytokines and permissive vasculature, hot tumors tend to exhibit a high number of neoantigens, which are novel peptide epitopes caused by mutations in the cancer genome. Non-synonymous mutations are changes in the cancer genome that produce an altered amino acid sequence that can drive tumorigenesis by altering cellular pathways or lead to expression of neoantigens<sup>[8]</sup>. Synonymous mutations do not change the amino acid sequence of an expressed gene but are not necessarily silent mutations. Synonymous mutations can serve as driver mutations by influencing translation, transcription, splicing, and mRNA transport<sup>[9]</sup>.

Melanoma and lung cancer are hot tumors that sometimes respond to immune checkpoint blockade<sup>[10,11]</sup>. Ultraviolet light in melanoma and smoke carcinogens in lung cancer induce DNA damage, and these tumors in older adults have accumulated higher numbers of non-synonymous mutations<sup>[8]</sup>. Pediatric cancers harbor few somatic mutations compared to adult tumors, and this is particularly true for pediatric brain tumors, which are almost always immunologically cold<sup>[12,13]</sup>. The lower tumor mutational load in pediatric brain tumors produces few neoantigens to stimulate T cell activation and proliferation within the tumor bed. Accordingly, an immunotherapy aimed at promoting an existing T cell immune response, such as checkpoint blockade, will be ineffective.

### DAMAGE RESPONSE AND TUMOR IMMUNITY

Inflammation is an important component of an immune response. While the CNS is not an immune-priv-

ileged site, regulatory immune cells and cytokines protect against excessive inflammation that would cause unacceptable inflammation involving the brain<sup>[14]</sup>. As brain tumors expand, local tissue damage and hypoxia induce regulatory cytokines and immune cells to quell inflammation and promote healing<sup>[15,16]</sup>. These factors contribute to the immunosuppressive behavior of the tumor itself and can blunt an anti-tumor immune response.

A typical endogenous immune response occurs in two phases. Pathogens, damaged DNA, cellular debris from apoptosis or necrosis, and inflammatory cytokines attract phagocytes, natural killer cells, and antigen-presenting cells as part of the innate immune response. Antigen-presenting cells then display peptide epitopes on MHC molecules, which engage T cells through their T cell receptor as part of the adaptive immune response.

Cells employ sophisticated DNA maintenance machinery to monitor and repair the genome, and damage-sensing pathways are important for eliminating pre-cancerous and cancerous cells<sup>[17]</sup>. Conventional chemotherapy and radiation, as well as innate-based immunotherapies, induce DNA damage and cell death by either apoptosis or non-apoptotic pathways<sup>[14,18,19]</sup>. Cell death and DNA degradation produce molecules called damage-associated molecular patterns (DAMP), which are recognized by the innate immune system and promote an immune response. DNA damage-sensing machinery within the nucleus transmits this signal to the cytoplasm and activates stimulator of interferon genes (STING) to induce proinflammatory interferon signals, which can shift the immunosuppressive tumor bed toward a more inflammatory, anti-tumor state<sup>[17]</sup>. DNA damage sensors also induce cell-surface ligand expression to recruit natural kill cells, natural killer T cells, and phagocytes to eliminate damaged cells and prime an adaptive immune response against the tumor<sup>[20,21]</sup>.

In health, damage-sensing pathways preserve the integrity of the genome and recruit the immune system to eliminate damaged cells when needed. In many instances, tumors deactivate the cellular damage-sensing machinery, which allows immune evasion and can dampen the response to conventional therapies like radiation or immunotherapies that are directly cytotoxic<sup>[22]</sup>. In addition, mutations within the damage-sensing machinery itself can contribute to tumorigenesis<sup>[22]</sup>. In this way, tumors with high mutational loads are more likely to harbor deleterious mutations within damage-sensing genes. This explains, in part, why hypermutated tumors are often resistant to radiation and alkylating chemotherapy<sup>[23]</sup>.

Defective damage response pathways have implications for immunotherapy as well. Innate-based immunotherapies are typically inflammatory and attempt to kill target cells to increase tumor antigen exposure. Tumor cells that lack damage-sensing machinery and have defective death pathways will be less amenable to many innate-based immune responses. The ultimate goal of any immunotherapy is to create a T cell response targeting the entire tumor and generates immunologic memory to protect against recurrence. With this understanding how mutational load, tumor neoantigens, and DNA damage machinery affect tumor immunology, we will discuss approaches in each of the main areas of immunotherapy for pediatric brain tumors.

## VIROTHERAPY

Virotherapy broadly refers to the use of viruses as therapeutic agents. Oncolytic viruses, which cause tumor cell death and can stimulate the immune system, are the most prominent clinical branch of virotherapy. Viruses are also useful as vectors for gene therapy, whereby viruses induce expression of a transgene that modifies the immune environment to promote an anti-tumor response. Retroviruses are used to genetically modify immune cells, most notably to express chimeric antigen receptors (CAR) on primary human T cells, and this will be discussed subsequently under adoptive cellular therapy. The last clinical branch of virotherapy, termed viral immunotherapy, uses viruses to introduce antigens that sensitize the host immune system

to the tumor through cross-reactivity or as an adjuvant. Currently, clinical applications of virotherapy in pediatric brain tumors are limited to approaches using oncolytic viruses or viruses as gene transfer platforms.

### Oncolytic viruses

Oncolytic viruses show promise in treating pediatric brain tumors. At least 50 clinical trials are ongoing using oncolytic viruses to treat cancer patients, mostly adults with non-CNS solid tumors. The oncolytic virus talimogene laherparepvec (TVEC), a modified type I herpes simplex virus for adults with advanced melanoma, is the first oncolytic virus to receive FDA approval<sup>[24]</sup>. Mechanistically, oncolytic viruses predominantly function through a combination of tumor cell lysis and stimulation of the innate immune system through DAMP and pathogen-associated molecular patterns (PAMP)<sup>[25]</sup>. Initially, oncolytic viruses were engineered for selective tumor tropism and direct cytotoxicity. However, oncolytic viruses are now regarded as immunotherapy for which efficacy depends on activating an endogenous anti-tumor immune response<sup>[26]</sup>.

TVEC enters tumor cells through nectin adhesion molecules and replicates within tumor cells that have dysfunctional anti-viral pathways<sup>[24]</sup>. The virus induces tumor cell death and causes DAMP and PAMP expression within the tumor. Additionally, the viral genome is genetically modified to increase MHC class I expression and to secrete GM-CSF, which promotes dendritic cell accumulation and antigen presentation to prime an adaptive immune response<sup>[24]</sup>. Taken together, this first-in-class agent represents how oncolytic viruses can be modified to stimulate innate immunity and readily combined with conventional and immune-based therapies. TVEC is undergoing clinical evaluation with checkpoint inhibitors and agents that target the MAP kinase pathway, which is activated in melanoma.

To date, at least five oncolytic viruses have been evaluated clinically in children with brain tumors: recombinant poliovirus<sup>[27,28]</sup>, adenovirus<sup>[29]</sup>, reovirus<sup>[30]</sup>, herpesvirus<sup>[31,32]</sup>, and new castle disease virus<sup>[33,34]</sup>.

PVSRIP0, is a recombinant, live attenuated, nonpathogenic oncolytic virus containing the oral poliovirus Sabin type 1 in which the internal ribosomal entry site (IRES) is replaced with the IRES from human rhinovirus. PVSRIP0 is administered intratumorally for adults with recurrent glioblastoma via convection-enhanced delivery and enters cells expressing the poliovirus receptor, CD155, which is ubiquitously expressed on malignant glioma. PVSRIP0 is directly cytotoxic and induces a marked inflammatory response. Interestingly, dendritic cells express CD155 and are infected by PVSRIP0. Whereas PVSRIP0 lyses tumor cells expressing CD155, the virus induces interferon-dominant activation of dendritic cells and tumor-specific CD8<sup>+</sup> T cells<sup>[35]</sup>. PVSRIP0 was well-tolerated in adults with recurrent glioblastoma (GBM) with some encouraging responses<sup>[36]</sup> and is being evaluated in a phase II trial in adults with recurrent GBM in combination with lomustine. PVSRIP0 is also being used to treat children with recurrent supratentorial malignant glioma as a phase Ib trial<sup>[28]</sup>.

Currently, three other early-phase trials using oncolytic viruses are ongoing for children with brain tumors. HSV G207, a modified type 1 herpesvirus, is delivered intratumorally to children with recurrent or progressive supratentorial malignant brain tumors. HSV G207 is cytotoxic and replicates within infected cells, then infects neighboring cells following cell lysis. Subsequent cohorts of patients will receive a single dose of radiation, which has been shown to increase viral activity in pre-clinical studies and was well-tolerated in an adult phase I trial<sup>[37]</sup>. Wild-type reovirus preferentially infects and kills cancer cells in its unmodified form<sup>[38]</sup> and induces an interferon-dominant immune response following intravenous administration in adults with brain tumors<sup>[39]</sup>. Reovirus is being evaluated in combination with GM-CSF in children with recurrent malignant brain tumors<sup>[30]</sup>. Newcastle disease virus (NDV) also induces selective tumor cell death and stimulates the innate immune system. NDV has been used clinically in cancer patients for decades with scattered clinical responses<sup>[40]</sup>. Currently, NDV is administered intratumorally in children with diffuse intrinsic brainstem glioma to induce tumor lysis. Tumor antigens are then harvested systemically and used to prime autologous

**Table 1. Virotherapy trials for pediatric brain tumors**

<b>Trial/therapy</b>	<b>Description</b>	<b>NCT/reference</b>
Recombinant poliovirus, PVSRIPO	Phase Ib trial evaluating PVSRIPO in children with recurrent supratentorial malignant glioma	NCT03043391 [27]
Modified type I herpesvirus, HSV G207	Phase I trial evaluating HSV G207 alone or with single radiation dose in children with recurrent supratentorial malignant brain tumors	NCT02457845 [32]
Wild-type reovirus	Phase I trial evaluating reovirus in combination with GM-CSF in children with recurrent malignant brain tumors	NCT02444546 [30]
Newcastle disease virus	Phase I trial evaluating newcastle disease virus in combination with autologous DC in children with brainstem glioma	[33]
Modified adenovirus, Ad-RTS-hIL-12	Phase I trial evaluating Ad-RTS-hIL-12, a vector for viral gene therapy, in children with progressive supratentorial tumors and diffuse intrinsic pontine glioma	NCT03330197 [47]

dendritic cells<sup>[34]</sup>.

### Viral gene therapy

Viruses are highly adept at introducing foreign genes and utilizing host machinery for protein expression. The human genome contains a large number of endogenous retroviral sequences, and roughly 8% of the human genome derives from infectious retroviruses<sup>[41]</sup>. Retroviruses, which predominantly infect dividing cells and stably integrate into the host genome, are useful for viral gene therapy. The lentivirus genus of retroviruses can transduce slowly-dividing or quiescent cells, overcoming in part the limitation that retroviruses must transduce dividing cells<sup>[42]</sup>. In cancer immunotherapy, retroviral transduction is typically performed *ex vivo* to genetically modify immune cells and not to deliver a direct therapeutic benefit.

Toca-511, however, uses a retroviral replicating vector to selectively transduce cancer cells with a yeast-derived cytosine deaminase gene following intratumoral administration. The prodrug 5-fluorocytosine is given systemically and selectively converted fluorouracil (5-FU) in tumor cells expressing cytosine deaminase. This platform illustrates how viruses are useful as a form of gene therapy and has shown clinical efficacy in adults with glioblastoma. Toca-511 has been studied preclinically in medulloblastoma models and has a strong rationale for clinical evaluation in children<sup>[43]</sup>.

Compared to retroviruses, adenoviruses more readily transduce non-dividing cells<sup>[44]</sup>. Adenovirus vectors typically have smaller DNA capacity and are more immunogenic, which can limit gene expression *in vivo*<sup>[45]</sup>. One adenovirus gene therapy platform is being evaluated clinically in children with brain tumors: The modified adenovirus, Ad-RTS-hIL-12, is injected intratumorally and uses an oral activator ligand to toggle IL-12 expression within the tumor. In early phase adult studies in recurrent GBM, this platform was well-tolerated, and preliminary data showed correlation between tumor response and IL-12 secretion<sup>[46]</sup>. Ad-RTS-hIL-12 is being evaluated in children with progressive supratentorial tumors and diffuse intrinsic pontine glioma<sup>[47]</sup>. Table 1 lists notable virotherapy trials for pediatric brain tumors.

### MONOCLONAL ANTIBODIES AND IMMUNOCONJUGATES

Similar to oncolytic viruses and tumor-directed viral gene therapy, monoclonal antibodies (mAb) and immunoconjugates can be used as immunotherapy to elicit an innate immune response. mAb directed against tumor-specific antigens have varied mechanisms of action, which are often incompletely understood, even in clinically effective products. For example, the HER2-specific mAb trastuzumab improves survival in patients with advanced HER2-positive breast cancer and is FDA approved in this disease<sup>[48]</sup>. Several anti-tumor mechanisms of trastuzumab have been identified, including antibody-dependent cell-mediated cytotoxicity (ADCC) involving Fc receptors on phagocytes<sup>[49]</sup>, inhibition of HER2 signaling<sup>[50,51]</sup>, and downregulation of HER2 cell-surface expression<sup>[51]</sup>.



There are two main ways mAbs can promote an immune response: ADCC and immune modulation. MAbs can trigger ADCC, in which cells of the innate immune system, specifically NK cells and phagocytes, lyse a tumor cell coated with antibodies containing Fc regions. There are no mAbs for primary brain tumors that function primarily by ADCC. Bevacizumab is a mAb that inhibits angiogenesis by binding to vascular endothelial growth factor (VEGF), a pro-angiogenesis cytokine. Bevacizumab is not strictly an immunotherapy but may have immunomodulatory effects, such as enhanced T cell recruitment and dendritic cell maturation and migration related to an inhibitory VEGF effect<sup>[52]</sup>. Bevacizumab is effective in some children with recurrent low-grade glioma<sup>[53]</sup> and FDA approved for recurrent GBM in adults<sup>[54]</sup>, but is not immunotherapy in the sense that it does not elicit an anti-tumor immune response. Similarly, monoclonal antibodies recognizing epidermal growth factor receptor (EGFR), block EGFR signaling in tumor cells and may, to a lesser extent, promote ADCC. Erlotinib, an anti-EGFR mAb, is ineffective against recurrent pediatric malignant glioma<sup>[55]</sup> and ependymoma<sup>[56]</sup>. Newer generation EGFR mAbs have been developed with improved ADCC characteristics but have not been evaluated in pediatric brain tumors<sup>[57]</sup>.

### Immunomodulatory monoclonal antibodies

MAbs can also promote anti-tumor immunity through immune modulation. Checkpoint inhibitors are an example of this and will be discussed separately. CD40 is a TNF receptor superfamily member expressed broadly on dendritic cells, B cells, and monocytes, as well as some tumor cells. Binding to the natural ligand CD40L expressed on T helper cells causes immune cell activation<sup>[58]</sup>. Agonistic CD40 mAbs activate antigen presenting cells and cytotoxic myeloid cells<sup>[59]</sup> and have induced clinical responses in adults with lymphoid tumors<sup>[58]</sup>. The CD40 agonistic antibody APX005M, delivered intravenously, is being evaluated in a phase I trial for children with recurrent malignant brain tumors and newly diagnosed diffuse intrinsic pontine glioma<sup>[60]</sup>.

CD47 is an integrin-associated protein ubiquitously expressed on human cells. In the context of tumor immunology, CD47 serves as a “don’t-eat-me” signal for macrophages<sup>[61]</sup>. The anti-CD47 mAb Hu5F9-G4 currently is in early phase trials for adults with lymphoid and non-CNS solid tumors. While not yet used clinically for primary brain tumors, Hu5F9-G4 has shown promising preclinical activity *in vivo* against orthotopic xenograft models of malignant pediatric brain tumors<sup>[62]</sup>.

### Immunoconjugates

Several immunoconjugates have reached the clinic in pediatric brain tumors as a form of immunotherapy. Immunoconjugates consist of an antibody fragment joined to some sort of effector molecule. Examples of effector molecules include immunotoxins, radioisotopes, and immune ligands. Immunoconjugates using immunotoxins are most prevalent and are typically comprised of a toxin coupled to a single-chain variable-region antibody fragment (scFv) that binds a tumor antigen. As a class, immunoconjugates typically have a short half-life following administration and are given directly into the tumor by convection enhanced delivery.

The EGFR gene is frequently amplified in adult GBM but not in pediatric GBM<sup>[63]</sup>. However, most pediatric glial tumors overexpress EGFR, making it an attractive target for immunotherapy for pediatric brain tumors<sup>[63]</sup>. D2C7-IT is an immunoconjugate comprised of a scFv that recognizes for both wild-type EGFR and the deletion variant EGFRvIII fused to the pseudomonal exotoxin PE38KDEL<sup>[64]</sup>. Upon binding EGFR, D2C7-IT is internalized and inhibits protein synthesis and causes tumor cell death. In a phase 1 trial in adults with malignant glioma, D2C7-IT induces inflammation within the tumor bed and has produced some clinical responses<sup>[65]</sup>. D2C7-IT will be evaluated in a phase I trial in children with recurrent, EGFR-positive malignant glioma at Duke.

Podoplanin is a tumor-associated glycoprotein highly expressed on pediatric malignant glioma and medulloblastoma<sup>[66]</sup>. A recombinant anti-podoplanin immunotoxin containing the pseudomonal exotoxin is effec-



**Table 2. Immunomodulatory mAb and immunoconjugate trials in pediatric brain tumors**

<b>Trial/therapy</b>	<b>Description</b>	<b>NCT/reference</b>
Agonistic CD40 mAb, APX005M	Phase I trial for children with recurrent malignant brain tumors and newly diagnosed diffuse intrinsic pontine glioma	NCT03389802 [60]
B7-H3 directed radioisotope, 124I-8H9	Phase I trial for children with diffuse intrinsic pontine glioma	NCT01502917 [71]

tive in preclinical pediatric brain tumor models but has not reached the clinic<sup>[66]</sup>. Immunoconjugates bearing a pseudomonal exotoxin that the IL-4 receptor<sup>[67]</sup>, IL-13 receptor<sup>[68]</sup>, or tumor growth factor alpha (TGFα)<sup>[69]</sup> were safe following direct administration into the tumor and intermittently effective in early-phase studies but have not been evaluated in children.

Immunoconjugates can also induce tumor death and anti-tumor immunity using radioisotopes, referred to as radioimmunotherapy. To date, only one radioisotope immunoconjugate has been used to treat primary pediatric brain tumors. <sup>124</sup>I-8H9 contains a scFv recognizing the B7-H3 antigen, expressed on glial tumors but not healthy cells, coupled to a radioactive iodine isotope<sup>[70]</sup>. <sup>124</sup>I-8H9 is being evaluated as a phase I trial for children with diffuse intrinsic pontine glioma and is administered intratumorally<sup>[71]</sup>.

Lastly, immunoconjugates incorporating an immune-activating ligand, also called bispecific antibodies, are being explored. Blinatumomab is a bispecific molecule that recognizes CD19, expressed on immature B cells, and CD3, which engages T cells. This T cell-engaging molecule induces remission in relapsed, immature B-lineage leukemia and is FDA approved for this disease<sup>[72]</sup>. Investigators at Duke developed a fully human, bispecific antibody (hEGFRvIII-CD3 bi-scFv) that redirects human T cells to kill EGFRvIII-positive malignant glioma cells<sup>[73]</sup>. This product is being evaluated in adults but is less useful for children with malignant glioma, as less than 5% of malignant glioma in children expresses EGFRvIII<sup>[74]</sup>. Table 2 lists current immunomodulatory mAb and immunoconjugate trials in pediatric brain tumors.

## IMMUNE CHECKPOINT INHIBITORS

Initial clinical evaluation of PD-1 and PD-L1 monoclonal antibodies demonstrated response rates of around 25% in adults with relapsed/refractory solid tumors<sup>[75,76]</sup>. Patients rarely had complete or sustained responses, but these encouraging results prompted evaluation checkpoint inhibitors in brain tumors. Unfortunately, these agents have been largely ineffective for most patients with brain tumors<sup>[77]</sup>.

A growing number of immune checkpoints are being targeted clinically, but mAbs targeting the PD-1/PD-L1 immune checkpoint remain the most widely used. Activated T cells express PD-1, a member of the CD28 family which impairs T cell activation and promotes T cell anergy and apoptosis<sup>[78]</sup>. PD-L1, which is expressed ubiquitously on solid tumors and also on regulatory immune cells within the tumor bed, binds PD-1 to dampen an anti-tumor T cell response<sup>[79,80]</sup>. Blocking this interaction using a mAb binding either PD-1 or PD-L1 can promote anti-tumor T cell activity.

In order for checkpoint inhibition to be optimally effective, the tumor must be immunologically hot, with T cell infiltration and tumor antigens that can be recognized by T cells. Tumor mutational load and T cell infiltration within the tumor are highly predictive for response with checkpoint inhibitors<sup>[81,82]</sup>. In melanoma and lung cancer, tumor mutational burden, which impacts the number of neoantigens and T-cell immunogenicity, correlate with response to checkpoint blockade<sup>[8,83]</sup>.

Hypermutant pediatric GBM, while rare, responds to anti-PD-1 checkpoint blockade. Two children with biallelic mismatch repair deficiency and hypermutated recurrent GBM responded to nivolumab<sup>[13]</sup>. These data are similar to those reported in adults with hypermutated colorectal carcinoma who received pembrolizumab, a PD-1 checkpoint inhibitor<sup>[84]</sup>.

Most often, pediatric brain tumors harbor fewer mutations compared to adult tumors, which have a lower mutational load than most solid tumors<sup>[13,85]</sup>. In a large analyses from over 300 adult glioma samples, less than 4% of tumors had a high tumor mutational load<sup>[86]</sup>. Even rare tumors that were hypermutated did not have significant T cell infiltration within the tumor. Taken together, these data explain at least in part why checkpoint blockade as monotherapy is unlikely to be impactful for pediatric brain tumors.

CheckMate 143 was a phase III randomized trial to evaluate efficacy of nivolumab, an anti-PD-1 monoclonal antibody compared to bevacizumab in adults with recurrent GBM. Nivolumab did not improve overall survival compared to bevacizumab<sup>[77]</sup>. Two additional trials of combining nivolumab and radiation with or without temozolomide in patients with newly-diagnosed, MGMT-unmethylated<sup>[87]</sup> and MGMT-methylated<sup>[88]</sup> GBM are ongoing.

While there have been no completed studies evaluating efficacy of checkpoint inhibitors in pediatric brain tumors, a number of trials are ongoing, including PD-1 antibodies as monotherapy or in combination with a CTLA-4 antibodies, and another checkpoint inhibitor against indoleamine (2,3)-dioxygenase (IDO). However, based on the disappointing results in CheckMate 143 and more recently for an IDO inhibitor in large phase III trial<sup>[89]</sup>, these agents are likely to be more effective in combination with immunotherapies which cause inflammation and promote T cell infiltration and activation first.

## ACTIVE IMMUNIZATION

Active immunization therapies deliver an immune stimulus to trigger an endogenous anti-tumor response. Typically, a vaccine is administered to stimulate and direct the host immune system to target antigens on the tumor. Cancer vaccines are a promising area of immunotherapy and are typically well tolerated. Vaccines containing tumor antigens, such as peptides, tumor lysate, or nucleic acids, and autologous dendritic cells are the most common approaches used clinically for patients with brain tumors. The intent of any active immunization strategy is to trigger an anti-tumor T cell response. T cell activation optimally occurs when T cells recognize antigen displayed on MHC molecules of antigen presenting cells in the setting of inflammation. Accordingly, active immunization approaches are designed to cause inflammation and antigen uptake by antigen presenting cells in lymphoid tissues, most often in lymph nodes.

### Dendritic cell vaccines

Dendritic cells (DC) are a critical link between the innate and adaptive immune systems. Upon encountering foreign antigens, specifically pathogen-associated molecular patterns, DC release inflammatory cytokines that activate the innate immune system. DC also process and present antigens to T cells and B cells, thereby activating naïve, effector, and memory immune cells or maintaining tolerance against self-antigens<sup>[90]</sup>.

Most commonly, DC for active immunization are generated by isolating monocytes from cancer patients that are expanded and activated *ex vivo*. These DC are loaded with either tumor lysate, peptides, nucleic acids, or viral epitopes that are expressed by the tumor. DC are usually matured with GM-CSF, then administered as a vaccine. Adjuvants such as tetanus toxoid are important to improve inflammation and immunogenicity in the host<sup>[90]</sup>.

Clinical testing of DC vaccines has demonstrated modest yet encouraging results in patients with advanced cancers<sup>[91,92]</sup>. There is general consensus that DC vaccines can induce tumor-specific T cell responses and immunological memory, and this is a promising platform for pediatric brain tumors<sup>[92]</sup>. To date, there have been several trials using autologous DC vaccines loaded with tumor RNA<sup>[93]</sup> or tumor lysate<sup>[94-96]</sup> for children with brain tumors. At this juncture, DCs are reliably manufactured and extremely well-tolerated. However, to improve efficacy, strategies to improve targeting, antigen loading, and migration *in vivo* are needed.

One of the central challenges for any active immunization approach is how to elicit an immune response against relatively weak “self” tumor antigens. Interestingly, cytomegalovirus (CMV) nucleic antigens are ubiquitously expressed in human malignant glioma<sup>[97]</sup>, and an adult patient treated with a DC vaccine pulsed with GBM tumor lysate developed a robust T cell response against the CMV antigen pp65<sup>[98]</sup>. The relative ease of eliciting an immune response against viral antigens contrasts with the difficulty of immunization against “self” tumor antigens and makes CMV an attractive target for immunotherapy. Dendritic cells targeting pp65 lead to long-term survival in small numbers of adults with newly diagnosed GBM<sup>[99]</sup>, and survival correlated with DC migration in a CCL3-depedant fashion<sup>[100]</sup>. This DC platform targeting CMV antigens will be evaluated in children with malignant glioma and recurrent medulloblastoma at Duke.

### Peptide vaccines

Manufacturing DC vaccines is costly, and poor DC migration following administration remains a challenge. Accordingly, active immunization strategies that stimulate endogenous DC activation are appealing, such as peptide vaccines, which inject tumor peptides with adjuvants, usually adjacent to lymph nodes. A few peptide vaccines for children with brain tumors are in early phase testing. One trial using a peptide vaccine targeting the H3.K27M neoantigen for HLA-A2+ children with H3K27M mutated glioma is underway<sup>[101]</sup>. A second peptide trial targeting the CMV epitopes pp65 and glycoprotein B is also underway for children with recurrent malignant glioma and medulloblastoma<sup>[102]</sup>. Additionally, a peptide trial using glioma-associated antigens for HLA-A2+ children with malignant brainstem and non-brainstem gliomas, including low-grade glioma, is underway<sup>[103]</sup>. This platform has been well tolerated and effective at generating an anti-tumor immune response<sup>[104]</sup>. At least four children with progressive, low-grade glioma have had sustained partial responses, providing evidence that peptide vaccines, typically given with Montanide adjuvant, can generate an endogenous anti-tumor response<sup>[105]</sup>. Montanide is a water-in-oil emulsion that acts as an adjuvant in these vaccines by enhancing CD4+ and CD8+ T cell response against antigens in the vaccine<sup>[106]</sup>.

Recently, highly personalized, neoantigen vaccines are gaining momentum. Initial clinical studies with cancer vaccines used whole tumor lysates, which contain a mixture of self-antigens and undefined neoantigens. These vaccines elicited broad immune responses but were generally ineffective. Using next-generation sequencing to identify DNA and RNA sequences of neoantigens and advanced algorithms to predict MHC I and MHC II loading, vaccines can be created that target specific neoantigens and hold promise for improving outcomes<sup>[92]</sup>. This personalized neoantigen approach was effective in some advanced melanoma patients, and combination with checkpoint blockade expanded the repertoire of neoantigen-specific T cells and further improved efficacy<sup>[107]</sup>. Table 3 lists notable past and current active immunization trials for pediatric brain tumors.

### ADOPTIVE CELLULAR THERAPY

Adoptive cellular therapy (ACT) involves manipulating effector immune cells *ex vivo* before transfer back to a patient with cancer. Initially, ACT for brain tumors used tumor-infiltrating lymphocytes (TIL) harvested from the tumor bed or immune cells isolated from peripheral blood or lymph nodes. Following collection, autologous lymphocytes were stimulated with cytokines or tumor antigen and infused back into patients. Overall, ACT using TILs or peripheral lymphocytes was well-tolerated but clinically ineffective, although immune activation and some responses were reported<sup>[108,109]</sup>. Natural killer T cells, which are specialized, CD1d-restricted T cells, recognize lipid antigens and have been tested in melanoma, but not brain tumors<sup>[110]</sup>.

By far, the most prominent type of adoptive cellular therapy involves cytotoxic T cells that are genetically modified to express a chimeric antigen receptor (CAR). CARs are synthetic receptors containing an antigen-binding domain, typically derived from the short chain variable fragment (scFv) of an antibody, coupled to the zeta chain and cytolytic machinery of a T cell receptor. Using retroviral vectors, primary human T cells

**Table 3. Notable past and ongoing active immunization trials for pediatric brain tumors**

<b>Trial/therapy</b>	<b>Description</b>	<b>Reference</b>
Monocyte-derived DCs loaded with tumor RNA	Phase I trial evaluating DCs pulsed with tumor RNA in children with brain tumors	[93]
Monocyte-derived DC loaded with whole tumor lysate	Phase I trial evaluating DCs pulsed with whole tumor lysate in children and adults with relapsed malignant glioma	[94]
Monocyte-derived DC loaded with whole tumor lysate	Phase I trial with DC pulsed with tumor lysate for children with newly diagnosed or recurrent high-grade gliomas	[95]
Monocyte-derived DC loaded with whole tumor lysate	Phase I trial with postoperative DC loaded with tumor lysate for children and adults with recurrent GBM	[96]
Peptide vaccine targeting H3.K27M	Phase I trial evaluating peptide vaccine targeting H3.K27M neoantigen for HLA-A2+ children with H3K27M mutated glioma	NCT02960230 [101]
Peptide vaccine targeting CMV epitopes pp65 and glycoprotein B	Phase I trial evaluating peptide vaccine targeting CMV pp65 and glycoprotein B for children with recurrent malignant glioma and medulloblastoma	NCT03299309 [102]
Peptide vaccine targeting glioma antigens	Phase I peptide trial using glioma-associated antigens for HLA-A2+ children with malignant brainstem and non-brainstem gliomas, including low-grade glioma	NCT01130077 [103]

are genetically modified to express the CAR molecule, which is designed to bind a tumor-restricted antigen and cause tumor cell death.

The CD19 CAR, which is effective against B-lineage lymphoid malignancies<sup>[111,112]</sup>, is FDA approved and induces remission in most patients with relapsed CD19-positive leukemia. CAR T cells targeting HER2<sup>[113]</sup>, IL13 $\alpha$ 2<sup>[114]</sup>, EGFRvIII<sup>[115]</sup>, and EphA2<sup>[116]</sup> have been used to treat adults with GBM. A trial involving CMV-specific cytotoxic T lymphocytes expressing a HER2 CAR treated seven children with GBM. There were no serious adverse events or instances of cytokine release syndrome, and at least one child had a partial response<sup>[113]</sup>. Transient responses following adoptive CAR T cell therapy are not infrequent, but almost all patients ultimately suffer disease progression.

There are multiple reasons the success of the CD19 CAR for B-lymphoblastic leukemia has not been duplicated by CAR T cells for brain tumors. The CD19 CAR targets an antigen that is ubiquitous and expressed solely on tumor cells or non-essential B cells without a strongly immunosuppressive tumor bed. Additionally, the CD19 single chain variable fragment (scFv) that guides the CAR T cell imparts an optimal activation profile and supports continued T cell killing<sup>[117]</sup>. This characteristic of the scFv is a key and unique distinction in this T cell product. ScFvs for other CAR T cells cause tonic signaling, which can cause T cell exhaustion and limits anti-tumor activity in patients following adoptive transfer<sup>[117]</sup>.

Antigen escape, tumor heterogeneity, and a harshly immunosuppressive immune microenvironment also contribute to treatment failure by CAR T cells. In a recently completed phase I trial for adults with recurrent GBM, EGFRvIII CAR T cells reliably reached the tumor bed following peripheral administration. However, *ex vivo* analyses from resected tumor showed dramatic adaptive resistance, with markedly increased PD-L1 expression and an influx of regulatory T cells, as well as decreased expression of the targeted EGFRvIII antigen<sup>[115]</sup>.

## CONCLUSIONS

Immunotherapy holds tremendous promise for improving outcomes for children with brain tumors. While checkpoint inhibitors and CAR T cells are well suited for hypermutated, immunologically hot tumors and B-cell malignancies, respectively, these modalities are less of a fit for pediatric brain tumors. Rather, immunotherapy approaches that induce inflammation and an innate immune response may be a better starting point, on which checkpoint agents and other T cell-directed agents can build.

While we are optimistic about immunotherapy in pediatric neuro-oncology, it is important to recognize that conventional chemotherapy and radiation will likely retain a role in treatment, particularly as both of these

modalities can be immunomodulatory and useful for shifting the immune balance toward anti-tumor immunity. Advanced surgical practice, radiation, and chemotherapy, including novel, targeted agents, remain important tools for treating our pediatric patients. It is important to point out that the most impactful treatment for brain tumors in the last decade is probably not an immunotherapy; BRAF and MEK inhibitors targeting the MAP kinase pathway, which is constitutively overactive in pilocytic astrocytoma and a fraction of other glial tumors, are radically changing how these diseases are treated and improving outcomes<sup>[118]</sup>. Taken together, the immunological context and molecular pathogenesis of each child's tumor must be considered on a case-by-case basis in determining any therapy, particularly in deciding what type of immunotherapy is most likely to add benefit.

## DECLARATIONS

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The authors declare that there are no conflicts of interest.

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Review

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# Therapeutic approach targeting apolipoprotein E binding region and low-density lipoprotein receptor for Alzheimer's disease

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## ABSTRACT

Approximately 13% of the population over the age of 65 years is estimated to have AD. The total number of cases is expected to increase over the coming decades. The apolipoprotein E (ApoE) genotype is the greatest genetic determinant for Alzheimer's disease (AD) development. The ApoE4 allele increases the risk of AD by 4 to 14 fold while the ApoE2 allele has an opposing effect; decreasing risk. Indeed many studies have demonstrated that carriers of the ApoE2 allele are associated with greater likelihood of survival to advanced age, superior verbal learning ability in advanced age, and reduced accumulation of amyloid pathology in the aged brain. In addition, it is known that ApoE proteins have different affinities for the low-density lipoprotein receptor (LDLR), with ApoE2 having the weakest binding to the LDL receptor at < 2% relative to ApoE3 and E4. Because ApoE2 has shown protective effects in regard to AD, a novel approach for ApoE4 carriers may be to create a peptide antagonist that blocks the ApoE interactions with LDLR at its 135-150 N-terminal binding domain. This peptide may create a more ApoE2-like structure by decreasing the affinity of ApoE4 for LDLR thereby reducing AD onset, memory impairment, and amyloid plaque formation. In this review, we will discuss the different detrimental effects that ApoE4 can cause. Most importantly, we will review how ApoE4 binding to LDLR promotes AD pathogenesis and how blocking ApoE4 binding may be a promising novel therapeutic approach for AD.

**Keywords:** Alzheimer's disease, low-density lipoprotein receptor, apolipoprotein E, amyloid precursor protein, late onset Alzheimer's disease



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## INTRODUCTION TO APOE

As life expectancies increase, more elderly patients are diagnosed with Alzheimer's disease (AD). AD brain exhibits close to 50% neuron loss in the cortex. Genome-wide association studies (GWAS) have identified the apolipoprotein E (ApoE) gene as a risk factor for AD<sup>[1,2]</sup>. It has been found that there is a strong correlation between ApoE4 carriers and higher levels of amyloid pathology. However, individuals who do not carry the ApoE4 allele seem to demonstrate fewer AD disease processes or other neurodegenerative disorders<sup>[3-6]</sup>.

The human ApoE gene is encoded on chromosome 19. ApoE is a 34-kDa protein consisting of 299 amino acids and is constitutively expressed in astrocytes, microglia, vascular smooth muscle cells, and choroid plexus while neurons typically generate ApoE under stress conditions. Through mRNA studies it has been demonstrated that the liver is the major producer of ApoE, followed next by the brain. The receptor-binding domain of ApoE is located within amino acids 136-150 of the N-terminal region. There are three different human isoforms of ApoE (ApoE2, ApoE3, and ApoE4) which differ by only 2 amino acids at sites 112 and 158. ApoE2 has cysteines located at both sites, ApoE3 has a cysteine at site 112 and arginine at site 158 while ApoE4 has arginines at both sites<sup>[7,8]</sup>. The heterogeneous nature of the three isoforms is secondary to genetic polymorphisms<sup>[9]</sup>. It has been shown that there is a linear reduction in brain hippocampal volumes by magnetic resonance imaging (MRI) scans according to ApoE genotype in the following hierarchy: ApoE4 < ApoE3 < ApoE2. In AD patients, ApoE4 carriers had significantly smaller hippocampal volume compared to ApoE2 carriers. This study used several well-characterized cohorts to analyze the neuroanatomic effect of ApoE on the left and right hippocampal volumes<sup>[10]</sup>. In addition, research has shown that the E4 allele is also a risk factor for atherosclerosis, human immunodeficiency virus (HIV) disease progression, cerebral amyloid angiopathy (CAA), tauopathies, dementia with Lewy bodies, and Parkinson's disease<sup>[11]</sup>.

## APOE4 MECHANISM

ApoE4 increases the risk of developing AD by 4-fold with one allele and 14-fold with two alleles<sup>[12]</sup>. The approximate allele frequencies of E2, E3, and E4 in the human population are 7%, 78%, and 14%, respectively<sup>[6,13]</sup>. Moreover, it has been shown through histological analyses of AD brains that ApoE is co-deposited with amyloid-beta (A $\beta$ ) in amyloid plaques<sup>[14]</sup>. It has also been revealed that A $\beta$  clearance is faster in ApoE3 transgenic mice versus ApoE4 transgenic mice<sup>[15]</sup>. This is likely because ApoE4 has an altered structure compared with ApoE2 or ApoE3, which alters its function. Therefore, understanding the structural properties of ApoE and its isoforms is vital to creating a prophylactic or therapeutic treatment. Research has shown that competition assays with ApoE4, ApoE3, and Tau revealed that ApoE4 inhibits Tau degradation. In addition, a single nucleotide polymorphism rs429358 defines ApoE4 and is located within exon 4 of apolipoprotein E. In regard to ApoE4, the arginine at position 112 directly influences arginine-61, which allows for domain interaction with glutamine-255. In addition, this bulky charged arginine residue destabilizes the N-terminal helix bundle domain, inducing helix shortening between amino acids 12 and 20 of the N-terminal domain and residues 204 and 210 of the C-terminal domain which reduces ApoE4 ability to form tetramers. This results in ApoE4 binding preference for very low-density lipoprotein (VLDL)<sup>[16-20]</sup>.

## APOE AND THE LOW-DENSITY LIPOPROTEIN RECEPTOR INTERACTION

LDLR is one member of a family of seven core LDL receptor-related proteins (LRPs), which also includes LDLR-related protein 1 (LRP1), the VLDL receptor (VLDLR), megalin (LRP2), apolipoprotein E receptor 2 (ApoER2), and LRP4. All LDL receptor family members share structural properties that allow interaction with ApoE<sup>[21]</sup>. In addition, LDL receptor family members contain a transmembrane domain which can be endocytosed, proteolytically processed, and interact with cell proteins, including direct interaction with (amyloid precursor protein) APP<sup>[22]</sup>. LDLR, VLDLR, LRP, and ApoER2 are present in a number of brain cells including astrocytes, microglia, neurons, and oligodendrocytes<sup>[23]</sup>. It has also been reported that overexpres-



sion of LDLR decreases ApoE levels in the brain, while LDLR deficient mice have increased ApoE brain accumulation<sup>[24,25]</sup>. Further, LDLR overexpression elevates uptake of A $\beta$  in astrocytes. Conversely, deletion of LDLR has an opposing effect<sup>[26]</sup>. Upon culturing brain sections with A $\beta$  plaques with murine astrocytes, A $\beta$  was taken up and degraded via LDL receptor or LDL receptor related protein<sup>[27]</sup>. ApoE contains 299 residues and was identified as a main component of lipoproteins in plasma. It has been established that lysine and arginine residues situated between ApoE residues 136 and 150 interact directly with acidic residues in the ligand binding domain of LDLR. In addition, full receptor binding activity requires arginine at position 172 located at the hinge region that connects the N- and C-terminal domains. ApoE3 and ApoE4 bind to LDL receptors with high affinity, but the binding of ApoE2 is 50- to 100-times weaker<sup>[28]</sup>. These data suggest that ApoE4 confers the highest risk for AD pathology due to its increased affinity for LDLR.

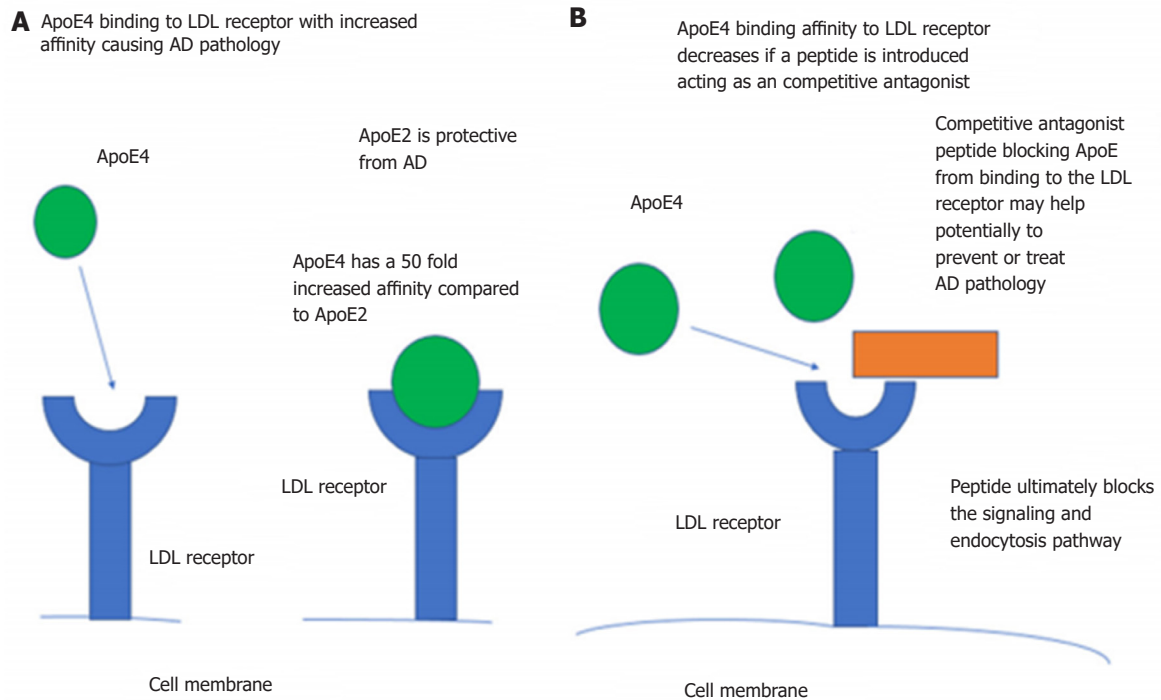
Recent research has shown that ApoE binding to ApoE receptors increases transcription of A $\beta$  through activation of the mitogen activated protein (MAP) kinase signaling pathway involving dual leucine-zipper kinase (DLK). In fact, ApoE binding to cell-surface ApoE receptors activates DLK. The levels of A $\beta$  potency production increase according to the different human ApoE isoforms (ApoE4 > ApoE3 > ApoE2). Specifically, when ApoE binds to ApoE receptor, DLK is activated. DLK will then activates dual specificity mitogen-activated protein kinase kinase 7 (MKK7) and extracellular signal-regulated protein kinase (ERK) 1/2 MAP kinases. Further more, activated ERK1/2 induces cFos phosphorylation, that will eventually stimulate the transcription factor activation protein (AP)-1. Transcription factor AP-1 will enhance transcription of APP and thereby increase A $\beta$  levels<sup>[29]</sup>. Therefore, a peptide or antibody blocking the interaction between LDLR and the ApoE binding site may potentially decrease the MAP kinase cascade and APP transcription, ultimately leading to a decrease in A $\beta$  production. Previous research demonstrated that the monoclonal antibody 1D7 is specific for human ApoE and blocks binding of lipid-associated ApoE to LDLR<sup>[30]</sup>. 2E8 monoclonal antibody also binds to ApoE and prevents ApoE-mediated binding of lipoproteins to the LDLR<sup>[31]</sup>.

## APOE2-LIKE PROPERTIES AND BENEFITS

Although ApoE2 known to cause type III hyperlipoproteinemia, the E2 allele is known for being protective against the development of late onset Alzheimer's disease (LOAD) compared to the common E3 and E4 allele as exemplified by a delayed age of onset and a greater likelihood of survival to advanced age. A cross-sectional multimodal neuroimaging approach has shown ApoE2 to be protective in the aged brain. In addition, the ApoE2 allele appears to have a relatively selective effect on reduced accumulation of amyloid pathology in the aged brain<sup>[32-34]</sup>. It has been reported that ApoE2 can promote type III hypercholesterolemia, leading to increased cardiovascular disease. However, studies demonstrate that ApoE4 knock-in mice have lower than normal brain cholesterol concentrations even though peripheral cholesterol levels are increased. This finding suggests that brain ApoE metabolism is distinct from that in the plasma. Moreover, the blood-brain barrier (BBB) effectively prevents the exchange of brain tissue and plasma lipoproteins. Thus, peripheral cholesterol cannot cross the BBB and enter the brain. Brain cholesterol is mainly synthesized in situ and provided by *de novo* synthesis, primarily by astrocytes and oligodendrocytes<sup>[11,32-35]</sup>.

ApoE2 is associated with slower cognitive decline, milder A $\beta$  pathology, and less neurodegeneration compared to ApoE3 and ApoE4. Older individuals who are ApoE2 carriers display superior verbal learning abilities, and faster processing of information. Possession of at least one copy of the ApoE2 allele has demonstrated a slower decline in episodic memory<sup>[34,36]</sup>. All isoforms of ApoE can modulate A $\beta$  clearance. However, aging APP transgenic mice expressing human ApoE2 also have the slowest rate of production of A $\beta$  oligomers with neuritic plaque formation compared to ApoE3 and ApoE4 mice<sup>[37]</sup>.

Rats expressing human ApoE2 have been shown to be protected from apoptotic death of cortical neurons induced by A $\beta$  peptides<sup>[38]</sup>. ApoE2 mice are also more effective in clearing A $\beta$  from the bloodstream and pro-



**Figure 1.** (A) ApoE4 has a 50 fold increased binding affinity to LDL receptor compared to ApoE2 (B). A novel approach is to create a peptide targeting the ApoE LDLR binding domain. This peptide can work as a competitive antagonist for patients who are ApoE4 carriers. Blocking the effect of ApoE4 binding affinity can help create a more ApoE2-like structure. ApoE2 is known to be protective in AD

moting degradation of A $\beta$ . In addition, ApoE2 carriers have increased dendritic outgrowth, which enhances the formation of new synapses and can protect against AD synaptic deterioration<sup>[34,39]</sup>. Further, ApoE2 protected cultured cells most effectively, compared to the other ApoE isoforms, from oxidative stress-induced death *in vitro*<sup>[40]</sup>. The cysteine to arginine substitution at position 158 in ApoE2 makes ApoE2 more stable to thermal and chemical denaturation, compared to ApoE3 and ApoE4. Moreover, the cysteine residue at position 112 creates a lesser chance to exhibit domain interactions relative to ApoE4<sup>[34,41,42]</sup>. It has been suggested that the development of drugs that can prevent the domain interaction of ApoE4 and convert ApoE4 to a more ApoE3/ApoE2-like structure may be beneficial for individuals with neurodegenerative disorders. In addition, a peptide blocking the 135-150 N-terminal region may create a more ApoE2-like structure, as ApoE2 has decreased affinity for the LDLR. Given that ApoE2 carriers have a lower risk and delayed age of onset of AD compared to E3 and E4 carriers<sup>[11,34,43]</sup>, it would stand to reason that creating a more ApoE2 structure can be beneficial for treating AD rather than using ApoE E3 or ApoE4 structures.

## CONCLUSION

Currently approximately 5.1 million Americans are affected with AD and the number is expected to triple by 2050. Further there are no truly effective disease-modifying therapies for AD. ApoE4 is known to play a major role not only in AD, but also atherosclerosis, CAA, tauopathies, dementia with Lewy bodies, and stroke. Approximately the allele frequencies of E2, E3, and E4 in the human population are 7%, 78%, and 14%, respectively. ApoE genotypes have different affinities for LDLR, with ApoE2 having the weakest binding to LDLR at ApoE3 > ApoE2<sup>[6,8,11,13,16,43-45]</sup>. We suggest that a peptide targeting the ApoE LDLR binding domain may work as a competitive antagonist for patients who are ApoE4 carriers, in effect creating a more ApoE2-like structure [Figure 1].

Creating a more ApoE2-like structure may be associated with greater likelihood of survival to advanced

age, superior verbal learning abilities, improved recall memory, faster processing of information, better test performance, and reduced accumulation of amyloid pathology in the aged brain. Furthermore, a second innovative approach would be to create a more advanced antibody targeting specifically the 133-152 N-terminal binding region of ApoE to prevent interaction between LDLR and ApoE. In sum, modulation of ApoE structure to create and/or enhance ApoE2-like activity may shed light on a novel approach for AD treatment and prevention.

## DECLARATIONS

### Authors' contributions

Reviewed the literature and wrote this article: Leon M

Edited and added further information to this article: Sawmiller D, Giunta B

Contributed to the initial idea of the review: Tan J

Read and approved this article: all authors

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Case Report

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# Cerebral fat embolism syndrome after long bone fracture due to traffic accident: a case report

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## Abstract

Cerebral fat embolism syndrome (CFES) is an uncommon but serious complication of long bone fracture. We reported a 19-year-old male patient who sustained CFES due to multiple limbs long bone fractures after a traffic accident injury. He gradually developed into coma within 24 h after his injury. The arterial blood gas analyses were normal. There was a small amount of gas in the right pleural cavity on the thoracic computed tomography (CT). Although there were no remarkable intracranial abnormalities on the initial brain CT findings, the typical brain magnetic resonance imaging (MRI) findings of the starfield pattern and scattered foci were observed. Both T2-weighted imaging and diffusion weighted imaging of MRI indicated multiple scattered lesions in the bilateral cerebrum hemisphere white matter, grey matter, basal ganglia, corpus callosum and thalamus indicative of acute infarcts without microbleeding on the susceptibility-weighted imaging sequences. With the above findings, the diagnosis of the case was cerebral fat embolism syndrome. Although the patient was treated with comprehensive support in the intensive care unit, he remained unconscious and was discharged after 7 days of hospitalization.

**Keywords:** Cerebral fat embolism, infarction, long bone fracture

## INTRODUCTION

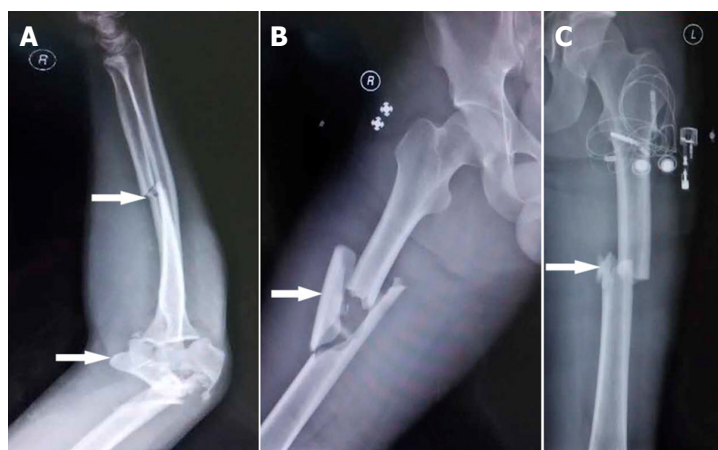
Fat embolism syndrome (FES) is a potentially fatal complication and occurs most commonly after long bone fracture. Many cases occur as subclinical events and remain undiagnosed. The incidence of clinically



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**Figure 1.** X-ray showed multiple long bone fractures, including right distal humerus distal, right ulna and its olecranon (A), bilateral femur shafts (B, C) (arrow)

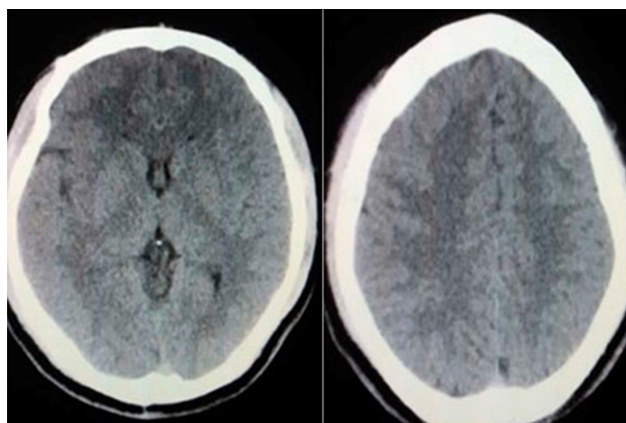


**Figure 2.** Thoracic computed tomography indicated a small amount of gas in the right pleural cavity (arrow)

significant FES occurs only in 0.9%-2.2% of long-bone fractures<sup>[1]</sup>. FES is characterized by various central nervous system, respiratory, cutaneous, and hematological manifestations<sup>[2]</sup>. Cerebral fat embolism syndrome (CFES) is a variant of FES. The neurological symptoms can be focal or diffuse, and most of the times exist with respiratory symptoms. We here reported a case of cerebral fat embolism (CFE) due to multiple long bone fractures of limbs after a traffic accident injury.

## CASE REPORT

A 19-year-old male patient was admitted to the Emergency Department after a traffic accident injury on May 19, 2016. He could not move his limbs except for the left upper limb due to bone fractures. He had no history of loss of consciousness, ear, nose, and throat bleed, vomiting, or convulsion. He was conscious, oriented with normal breathing and was hemodynamically stable. In the physical examination, body temperature was 36.8 °C, pulse was 78/min, breath rate was 22/min, and blood pressure was 130/65 mmHg. There were not positive neurologic dysfunctions. Electrocardiogram was normal. X-ray showed multiple long bone fractures, including bilateral femurs, right distal humerus, right ulna and its olecranon [Figure 1]. There was a small amount of gas in the right pleural cavity on the thoracic computed tomography (CT) [Figure 2].

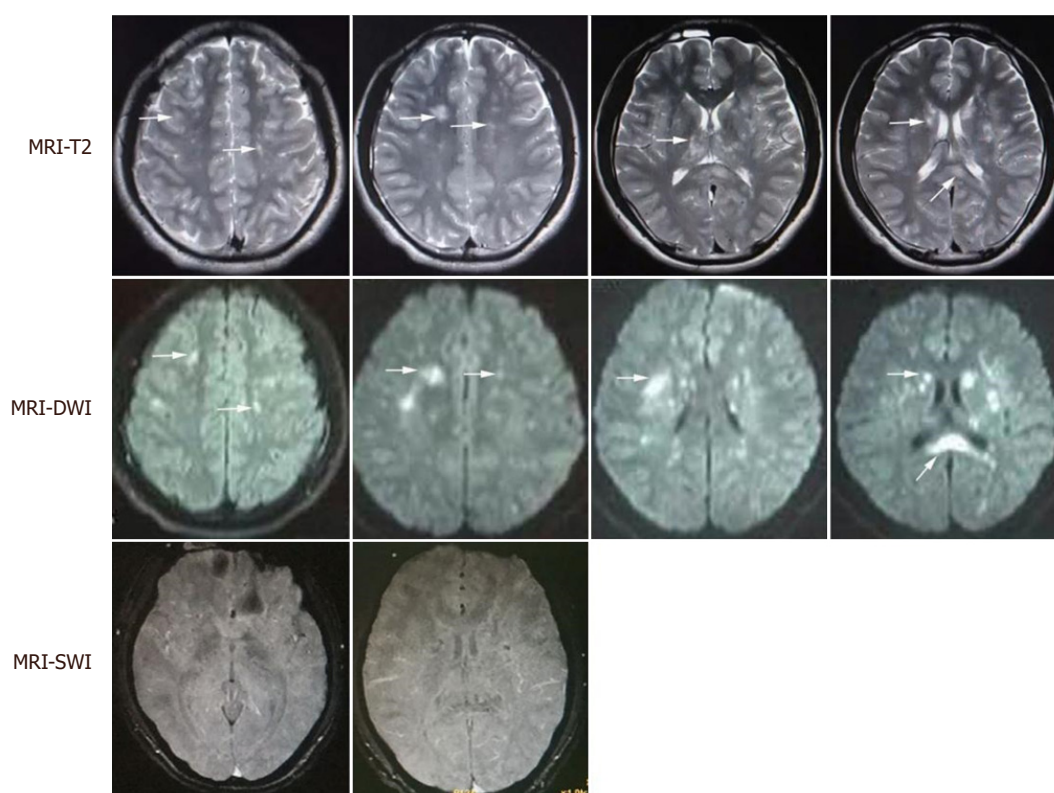


**Figure 3.** There was no remarkable abnormality on the brain computed tomography on admission

However, there were no remarkable abnormalities on abdominal CT and color Doppler ultrasound. The results of hematologic and biochemical parameters of the patient were as follows at first admission: hemoglobine 149 g/L, platelet count  $187 \times 10^9/L$ , procalcitonin 0.10 ng/mL, prothrombin time 13.7 s, alanine aminotransferase 196 U/L, aspartate aminotransferase 286 U/L, lactate dehydrogenase 739 U/L, creatine kinase 1092 U/L, creatine kinase MB 78 U/L, and cardiac troponin I 0.01 ng/mL. The patient did not have hypoxemia and the arterial blood gas analyses were normal. He was treated in the intensive care unit. About fifteen hours later, he became somnolence and gradually developed into a coma. Neurological examination revealed bilateral Babinski sign. His pupils were isocoric and bilaterally responsive to light. There were not cutaneous manifestations. Blood gas analysis was retested and normal. Echocardiogram showed normal ventricular function without any thrombus or patent foramen ovale (PFO). Bilateral lower limb vascular color Doppler ultrasound did not show any thrombus signs. Although in the present case there were no remarkable intracranial abnormalities on the initial brain CT findings [Figure 3], the typical brain magnetic resonance imaging (MRI) findings of starfield patterns were observed. Both T2-weighted imaging and diffusion weighted imaging of MRI indicated multiple foci lesions in the bilateral cerebrum hemisphere white matter, grey matter, basal ganglia, corpus callosum and thalamus indicative of acute infarcts [Figure 4]. However, there was not any microbleeding on the susceptibility-weighted imaging sequences of brain MRI [Figure 4]. With the above findings, the diagnosis of the case was cerebral fat embolism syndrome. The patient was treated with methylprednisolone injection (80 mg, intravenous infusion, twice a day) and low molecular weight heparin calcium injection (Dalteparin Sodium, Fragmin: 0.2 mL/5000 IU, subcutaneous injection, once a day), antibiotics (Cefoperazone sodium and sulbactam sodium) and dehydrating drugs (such as mannitol and human albumin solution). The bone fractures were firstly externally fixed upon admission into the hospital and then internally fixed on fifth day during in-hospital stay. Unfortunately, the patient did not recover and remained unconscious, and he was discharged after 7 days of hospitalization.

## DISCUSSION

FES is a common clinical entity that can occasionally have significant neurological sequelae. Fat embolism syndrome has been reported not only after long-bone injuries, but also after rib or tarsal bone involvement<sup>[3,4]</sup>. The risk of fat emboli is highest within the first few days after trauma. Clinical symptoms of fat embolism usually develop gradually within 24 to 72 h after injury<sup>[5]</sup>, but in some cases, early manifestation occurs. Patients can present with triad of varying severity of neurological, respiratory, and cutaneous manifestations, depending on the embolic burden in the respective vasculature. CFES is a variant of FES characterized by a predominance of neurologic manifestations often without the pulmonary or dermatologic findings seen in FES. The period from the time of injury to the development of cerebral FES is typically between 12 h and 3 days<sup>[6]</sup>.



**Figure 4.** Both T<sub>2</sub>-weighted imaging and diffusion weighted imaging (DWI) of magnetic resonance imaging (MRI) indicated multiple foci lesions in the bilateral cerebrum hemisphere white matter, grey matter, basal ganglia, corpus callosum and thalamus indicative of acute infarcts (arrow). However, there was not any microbleeding on the susceptibility-weighted imaging sequences of brain MRI

CFES occurs after fat emboli enter the arterial circulation. However, the underlying mechanism remains poorly understood. It was suggested that fat globules may enter the arterial circulation by two mechanisms<sup>[2,7]</sup>. Firstly, fat globules can enter the left atrium directly from the right heart through a shunt, such as a PFO (paradoxical embolism), especially when the patient takes a deep breath or performs Valsalva maneuver after trauma. Secondly, microglobules of fat may filter directly through the lung capillaries to reach the arterial system. The diameter of these microemboli are small (7-10  $\mu\text{m}$  in diameter) and malleable and may not lead to significant pulmonary injury<sup>[7]</sup>. In our case, the absence of PFO and arrhythmia in this patient with CFE supports the latter mechanism. However, the mechanisms underlying the pathophysiology of CFE are complex. Great amounts of microglobules of fat may randomly enter the internal carotid artery or/and vertebralbasilar artery, and usually cause distal vascular occlusion. Simple vascular occlusion with ischemia probably does not fully explain the FES. Mechanical mobilization of fat globules from the bone marrow to the intramedullary veins and pulmonary microcirculation, along with endothelial damage by fat metabolism products and cytokines, has been proposed<sup>[8]</sup>. Such biochemical pathways of injury result from the complex inflammatory response to free fatty acids released by the hydrolysis of embolized fat that has entered blood vessels. This inflammatory response may have local effects on the brain and other tissues and systemic consequences, including shock and anticoagulation<sup>[2,7]</sup>.

Neurologic manifestations of CFES vary greatly, ranging from mild headache, diffuse encephalopathy, and seizures to focal features, including pyramidal signs, pupillary paresis, and aphasia<sup>[1,2,7]</sup>. These signs can occur in isolation or accompany with respiratory and cutaneous manifestations. Some patients do not have hypoxemia, and these petechiae occur in only 20%-50% of patients and resolve quickly. Therefore, it is difficult for us to diagnose the case. The Gurd's and Wilson's criteria are often used to make diagnoses<sup>[9]</sup>.

One major and four minor criteria must be present to formally diagnose FES. Neither of these diagnostic tools includes brain imaging, which seems to be the most specific. CFES is a clinical diagnosis, but specific findings on neuroimaging studies can be strongly supportive. The purpose of a CT scan is to rule out certain stroke mimics and detect hemorrhage, not necessarily to rule in the diagnosis of ischemic stroke. CT scans may not be sensitive enough to detect an ischemic stroke, especially if it is small, acute (especially within 24 h of the stroke onset), or in the posterior fossa (i.e., brainstem and cerebellum areas). In other words, a normal CT scan does not rule out the diagnosis of ischemic stroke. Noticeably, it is important to underline that a careful examination of brain-CT findings, such as the topography and density measurements of round lesions (such as round hypodense lesions,  $-40$  HU)<sup>[3]</sup>, were enough to confirm the clinical suspicion of CFE<sup>[3]</sup>. This is important because in some cases the MRI cannot be available or would be impossible to perform. MRI is more sensitive and demonstrates multiple small hyperintense, intracerebral lesions. There were great amounts of hyperintense lesions on MRI T2-weighted scans. The most characteristic MRI finding is the starfield pattern, demonstrating scattered foci of high-intensity restricted diffusion on diffusion-weighted imaging<sup>[7,10]</sup>. This is most apparent in the acute phase, from 4 h to the first few days from the time of injury. Such widespread petechial hemorrhage and bland microinfarction have been demonstrated on autopsy<sup>[11]</sup>. In our case, the initial brain-CT scan was normal, while MRI showed extensive cortical and subcortical regions fat embolism which led to disturbance of consciousness. There are few differential diagnoses of disseminated hyperintense lesions on T2-weighted scans which include diffuse axonal injury, areas of vasogenic edema associated with microinfarcts, and demyelinating diseases<sup>[12]</sup> which were ruled out by history and clinical scenario, in our case. Of note, in some patients who sustained severe trauma, both CFE and diffuse axonal injury (DAI) could be the cause of altered consciousness in the absence of marked intracranial lesions in cranial CT<sup>[13]</sup>. However, distinguishing CFE and DAI can be difficult clinically. Generally, DAI develops immediately after the insult, whereas CFE occurs 24 to 72 h after the trauma and even after internal fixation for the fractures<sup>[13]</sup>. It was reported that there was no significant difference between diagnostic performance of diffusion tensor imaging (DTI) and conventional MRI in CFES, but a difference in directional diffusivities was clearly identified between CFES and DAI.

There are currently no disease-specific treatment guidelines for FES or CFES other than supportive care to address both intrinsic lung pathology and airway protection in the setting of neurological impairment<sup>[2]</sup>. Pharmacological intervention, including administration of heparin, dextran, aspirin, statin, albumin, and steroids and glucose loading, proved to be ineffective<sup>[14,15]</sup>. Corticosteroids have been extensively studied with variable results, and their use is controversial. In cases of fulminant FES, corticosteroids may be considered. Gupta *et al.*<sup>[16]</sup> propose a regimen of methylprednisolone 1.5 mg/kg IV every 8 h for 6 doses in a select group of patients with long bone or pelvic fractures at high risk of developing FES and without significant contraindications. In our case, the patient was treated with methylprednisolone injection (80 mg twice a day). The side effects of corticosteroids such as promoting coagulation and ulcer, disorder of electrolyte metabolism should be emphasized. Anticoagulation has been shown to prevent stroke in patients with cardioembolic and other noncardioembolic sources. However, the early use of anticoagulants has been associated with hemorrhagic transformation. Hitherto, there is insufficient evidence to support that routine administration of anticoagulation agent is effective and safe for FES. One possible benefit from anticoagulation for fat embolism may potentially decrease the risk of deep vein thrombosis. Early surgical stabilization should be considered. Early fixation of fractures within 24 h has been recommended to prevent further trauma at the injury site, thus decreasing the incidence of FES. The prognosis of CFES is variable, depending on the severity of the manifestations and on the quality and timing of treatment. Most of patients recovered fully from this disease, and other survivors remained in cognitive disorder, and some even die<sup>[3,7,15]</sup>.

In summary, we highlight that CFES could develop within hours after long bone fractures. Neurologic manifestations of CFES vary greatly. Neuroimaging is critical in the diagnosis of CFES. The brain-CT scan indicating the presence of round, hypodense lesions within the range of fat ( $-40$  HU) suggests fat embolism.



The typical starfield pattern may be present on MRI in some cases of acute phase. Supportive respiratory and neurological cares remain the mainstay of therapy. Prognosis is variable depending on the context of concomitant illness and premorbid functional status.

## DECLARATIONS

### Authors' contributions

Conception and design of study, first draft and revision of manuscript: Chen XY, Fan JM

Provided some clinical data: Deng MF

Provided the images data: Jiang T, Luo F

Read and approved the final manuscript: all authors

### Availability of data and materials

The data and material could be available to readers upon request.

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### Conflicts of interest

All authors declare that there are no conflicts of interest.

### Ethical approval and consent to participate

The study was approved by the local ethics committee and informed consent was obtained from the patient's guardian.

### Consent for publication

Not applicable.

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Review

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# Systemic therapy in patients with NSCLC with brain metastasis: the emerging role of immunotherapy

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## ABSTRACT

Brain metastasis (BM) is very common in advanced non-small cell lung cancer (NSCLC) patients. The development of BM remains a serious complication associated with significant morbidity and mortality. The traditional approach has been largely focusing on local therapy with surgery and/or radiation. New approaches to treat BM in NSCLC are urgently needed to offer safe and effective therapy as well as to preserve neurocognitive function. There has been significant progress in development of systemic therapies to treat advanced NSCLC in recent years. Targeted therapy has been gradually incorporated into clinical practice to manage NSCLC with BM. Immunotherapy (IO) has revolutionized our treatment paradigm to manage advanced NSCLC. In this review we outline the systemic options for NSCLC-related BM and discuss IO in NSCLC. Finally, we describe the available data and future perspective to support the use of IO in NSCLC patients with BM.

**Keywords:** Immunotherapy, non-small cell lung cancer, brain metastasis

## INTRODUCTION

An estimated 1.8 million new lung cancer cases occurred worldwide in 2012, accounting for approximately 13% of total cancer diagnoses. Lung cancer is the leading cause of cancer mortality in both men and women in developed countries<sup>[1]</sup>. Roughly 85% of lung cancers are non-small cell lung cancer (NSCLC) and the 5-year survival rate in stage IV remains less than 5%<sup>[2]</sup>. Brain metastasis (BM) has long been recognized to represent a common event in NSCLC. About 10%-25% of NSCLC patients present with BM upon the initial



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diagnosis, and up to 50% will develop BM at some point during the course of the disease<sup>[3,4]</sup>. The incidence of BM appears to have increased in the past decade due to the wide use of magnetic resonance imaging to detect smaller lesions as well as to the evolution of more effective systemic therapy leading to better control of extra-cranial disease and prolonged survival<sup>[5,6]</sup>. Historically, the development of BM is associated with poor prognosis with median overall survival (OS) ranging from 3.4 to 7.1 months after whole brain radiation therapy (WBRT)<sup>[7]</sup>.

## LOCAL THERAPY

Local therapy with surgery and/or radiation therapy has been the mainstay of treatment for decades. For younger patients with a newly diagnosed solitary BM, surgical resection followed by WBRT is supported by level I evidence and incorporated in some clinical guidelines<sup>[8]</sup>. WBRT has been the standard of care for NSCLC patients with multiple BM. This practice was recently called into question. In the QUARTZ trial, WBRT failed to show any survival or quality of life benefit compared with steroid and best supportive care in NSCLC patients with multiple BM, although majority of the patients had poor performance status (PS)<sup>[9]</sup>. On the other hand, WBRT was deemed inadequate in the long-term control of BM in patients with good PS and prolonged survival due to effective systemic therapy. Moreover, these patients may survive long enough to experience significant neurocognitive sequelae associated with WBRT. To avoid neurotoxicity from WBRT, stereotactic radiosurgery (SRS) has been advocated in patients with better prognosis and a limited number of BM. A meta-analysis demonstrated that WBRT with or without SRS did not improve OS compared with SRS alone in patients with up to four BM; whereas SRS was substantially superior in preserving PS and neurocognitive function<sup>[10]</sup>. A Japanese study of SRS alone for patients with one to ten BM showed that distant brain failure rate and survival did not differ significantly in patients with two to four versus five to ten lesions<sup>[11]</sup>. In patients with BM who underwent resection, SRS to the surgical cavity was compared head-to-head with WBRT in a randomized phase 3 study. It demonstrated no difference in OS, but less frequent decline in cognitive function (52% vs. 85% at 6 months) in SRS group. Thus, SRS is regarded as one of the standards of care after resection of a brain metastasis<sup>[12]</sup>. For appropriate patients, SRS has been getting increasingly utilized in practice in recent years.

## CHEMOTHERAPY

For years the role of systemic therapy in treating BM in NSCLC patients has been neglected owing to the prevailing belief that therapeutic drugs cannot cross the blood brain barrier. Traditional chemotherapy with platinum-based doublets has been shown in the first-line setting to be active in controlling BM in small studies. The commonly used regimens for adenocarcinoma of a platinum plus pemetrexed were shown in phase 2 studies to demonstrate roughly 40% intracranial response rate (ICRR), comparable to systemic response rate<sup>[13,14]</sup>.

## TARGET THERAPY

With recent advances in understanding NSCLC biology, many highly specific target agents have become available, redefining the role of systemic therapy in treating BM in NSCLC. In recent years, several distinct molecular driver mutations have been discovered, and the list is getting longer over time. The most common epidermal growth factor receptor (EGFR) mutation constitutes 10%-15% of lung adenocarcinoma in the Western population<sup>[15]</sup>. Early-generation tyrosine kinase inhibitors (TKIs) have poor penetration to the brain, although they were shown to have variable success in treating BM in a pool analysis<sup>[16]</sup>. Weekly intermittent “pulsatile” administration of high-dose erlotinib (1500 mg/week) in a small retrospective analysis of 9 patients exhibited an ICRR of 67%, with median time to progression of merely 2.7 months<sup>[17]</sup>. The 3rd-generation TKI represented by osimertinib, was designed to overcome resistant EGFR mutation T790M and was found to have better permeability in brain. In a pooled analysis of two phase 2 trials, 50 patients with

T790M positive NSCLC with BM were treated with 80 mg osimertinib. The ICRR was 54% (12% CR) and the brain disease control rate (DCR) was 92%; 75% of patients were still in response at 9-month follow-up<sup>[18]</sup>. Osimertinib showed very impressive PFS as well as intracranial control over gefitinib or erlotinib in newly diagnosed NSCLC with EGFR mutation and has been approved for first-line use<sup>[19]</sup>.

Anaplastic lymphoma kinase (ALK) rearrangement defines another subset of mutations in 3%-5% of NSCLC. BM has been reported in 24% of patients upon diagnosis and up to 70% during the course of disease<sup>[20,21]</sup>. It is not entirely clear that it is due to poor brain penetration of first-generation TKI, crizotinib, or that the brain is the preferential site of metastasis of ALK + NSCLC. Second-generation ALK TKIs such as ceritinib, brigatinib and alectinib all showed much better brain penetration and great activity against BM. In ALEX trial, a subset of ALK+ NSCLC patients with measurable CNS lesions at baseline were treated with alectinib *vs.* crizotinib. The ICRR and PFS were 81%, 50% and 17.3 months, 5.5 months, respectively<sup>[22]</sup>. Moreover, 45% of the patients with BM (measurable or nonmeasurable) treated with alectinib achieved CR<sup>[22]</sup>. Thus, alectinib represents a great treatment option for patients with ALK + NSCLC presenting with BM.

## IMMUNOTHERAPY AND NSCLC

Immune checkpoint inhibitors have transformed the treatment paradigm for advanced-stage NSCLC in both frontline and platinum-resistant settings. These agents inhibit a key inhibitory signaling pathway in T cell activation by blocking programmed cell death a (PD-1) from binding to the ligand 1 or 2 (PD-L1/PD-L2), thereby allowing activation of the inactivated T cells<sup>[23]</sup>.

### Immunotherapy as first-line treatment

In the pivotal Keynote 024 study, 305 treatment naïve, PD-L1 positive (tumor proportion score, TPS  $\geq$  50%) patients with advanced-stage NSCLC without EGFR, ALK mutations were randomized to receive pembrolizumab, an anti-PD1 monoclonal antibody *vs.* platinum-based chemotherapy. Pembrolizumab was associated with higher ORR (44.8% *vs.* 27.8%) and prolonged median PFS (10.3 months *vs.* 6.0 months)<sup>[24]</sup>. Based on this study, the FDA approved the use of pembrolizumab in PDL1 positive patients with advanced-stage NSCLC in frontline setting.

Combinations of immunotherapy with chemotherapy or another immunotherapy agent have also been extensively studied in the past few years. Keynote 189 was another landmark study that confirmed the result of the previously published small phase 2 study on first-line chemo-immunotherapy with carboplatin/pemetrexed/pembrolizumab in previous untreated non-squamous NSCLC patients<sup>[25,26]</sup>. 616 patients were randomly assigned in a 2:1 ratio, to receive pemetrexed and a platinum-based drug plus either pembrolizumab or placebo every 3 weeks for 4 cycles, followed by pembrolizumab or placebo plus pemetrexed maintenance. The Pembrolizumab-combination group was demonstrated to have a better RR (47.6 % *vs.* 18.9%), a longer PFS (8.8 months *vs.* 4.9 months) and median OS (not reached *vs.* 11.3 months). The benefit was seen in all subgroups, including those with a PD-1 TPS < 1%. The RR was highest in patients with PD-1 TPS  $\geq$  50% (61.4% *vs.* 22.9%)<sup>[26]</sup>. Thus, there are two FDA-approved options for NSCLC in the first-line setting at this time. For metastatic squamous NSCLC, platinum/taxane with or without pembrolizumab was investigated in Keynote 407 study. The ORR was 58.4% in the pembrolizumab-chemotherapy group compared with 35% in the chemotherapy group. OS was 15.9 months *vs.* 11.3 months, respectively. PFS was observed in all PD-L1 TPS subgroups, although the PD-L1 high subgroup had a much longer PFS (8 months *vs.* 4.2 months). The platinum/taxane/pembrolizumab combination represents a new standard of care in metastatic squamous NSCLC<sup>[27]</sup>. Immuno-immunotherapy combination is also under intense investigation. In the Checkmate 227 study, ORR and PFS among patients with a high tumor mutational burden (TMB) defined by at least 10 mutations per megabase was 45.3% and 7.2 months in the immuno-immunotherapy combination group with nivolumab plus ipilimumab irrespective of PD-L1 expression level. The response was more durable, with 68% of patients having an ongoing response after 1 year with combination group *vs.* 25% with chemotherapy<sup>[28]</sup>.

Thus, nivolumab plus ipilimumab may represent a new treatment option in metastatic NSCLC with high TMB, though this combination has not been approved to be used clinically.

### Immunotherapy as second-line treatment

Currently there are three agents available in the platinum-resistant setting. Two large phase 3 randomized clinical trials made head-to-head comparison between nivolumab and docetaxel<sup>[29,30]</sup>. Checkmate 057 included patients with non-squamous histology, and Checkmate 017 included patients with squamous histology with identical trial design<sup>[29,30]</sup>. Both trials showed superiority of nivolumab in ORR, PFS and OS as well as significant improvement in quality of life<sup>[29,30]</sup>. Nivolumab was reported to benefit both PD-L1 negative and positive groups with a different antibody, and was approved for 2nd-line use irrespective of PD-L1 expression based on these two trials. Pembrolizumab was compared with docetaxel in the Keynote 010 study wherein it only included PD-L1 positive patients defined as TPS  $\geq 1\%$ . The pembrolizumab arm showed significantly better ORR, PFS, OS with a very favorable side effect profile<sup>[31]</sup>. Thus, pembrolizumab was approved in PD-L1 positive NSCLC after platinum-based first-line therapy. Atezolizumab, a monoclonal antibody against PD-L1, was compared directly with docetaxel in a phase 2 and a phase 3 randomized trial with similar results. Although there was no median PFS advantage in the atezolizumab arm, an OS advantage was found in all subgroups, which led to the addition of atezolizumab to the armamentarium against NSCLC in the second-line setting<sup>[32,33]</sup>. The landscape of immunotherapy for NSCLC is rapidly changing. These three agents were all approved before first-line immunotherapy became available. With expected wide use of mono-immunotherapy or chemo-immunotherapy combination in the frontline, there is unmet medical need for immunotherapy to overcome immune resistance in previously treated population.

### Immunotherapy in NSCLC with brain metastasis

The brain was once believed to be an immune privileged organ to actively suppress any immune response. It is now known that the immune response in brain parenchyma is tightly regulated<sup>[34]</sup>. In established brain metastases, the tumor microenvironment is composed of the innate immune system, namely microglia and blood-derived myeloid cells/macrophages; and the adaptive immune system, mainly represented by T cells<sup>[34,35]</sup>. Brain metastases were shown to have higher PDL-1 expression and lower density of tumor-infiltrating lymphocytes (TILs) than the matched NSCLC primary tumors<sup>[36]</sup>. The immunogenicity of the primary tumor probably influences the T cell response in brain metastases as denser TILs were observed in melanoma-derived brain metastases followed by renal cell carcinoma and NSCLC<sup>[37]</sup>. The density of TILs seems to correlate well with extent of brain edema and OS<sup>[37]</sup>. Therefore, the preconditions in the tumor microenvironment to respond to immunotherapy are present in BM.

Patients with active BM from NSCLC have been excluded from pivotal clinical trials of immunotherapy in NSCLC, thus there is no high-level evidence available from the prospective studies. However, in the Keynote 24, the Keynote 189 (trials of pembrolizumab in the frontline), and the OAK trial (atezolizumab in the second-line setting), patients with previously treated brain metastasis were allowed to be enrolled. Specific intracranial response was not available, but the OS benefit seemed to have been equally sustained<sup>[26,32,38]</sup>. The expanded access program (EAP) of nivolumab in Italy included a cohort of 409 NSCLC patients with symptomatic or controlled BM. The real-world experience demonstrated ORR of 17% and DCR of 40% respectively, similar to the whole population of 1588 patients with median OS of 8.6 months and 11.3 months, respectively<sup>[39]</sup>. These results seem to suggest the overall benefit was able to be retained in the subgroup of patients with BM. However, it remains unknown from these trials to what extent the intracranial control derives directly from immunotherapy.

In a retrospective study, 43 NSCLC patients with BM were included, and 16 patients had active BM. Patients treated with nivolumab achieved an ICRR and an extracranial RR of 9% and 11%, respectively. The median intracranial and the general PFS were 3.9 months and 2.8 months, respectively<sup>[40]</sup>. Although the RR was rela-

tively low, these were real-world unselected cases without known PD-L1 status and the PFS was in line with pivotal immunotherapy trials in the 2nd-line setting. More importantly, the RR appears to be equivalent for both intracranial and extracranial disease.

The single-agent activity of pembrolizumab in the brain was also studied in a phase II trial including both NSCLC and melanoma patients with at least 1 BM between 5 and 20 mm that was asymptomatic and either untreated or progressing after prior local therapy. The result was recently updated at 2018 ASCO annual meeting<sup>[41,42]</sup>. Two cohorts of NSCLC patients were enrolled with 34 PD-L1 positive (PD-L1  $\geq$  1%) patients in cohort 1, and 5 PD-L1 negative or unevaluable patients in cohort 2. Pembrolizumab 10 mg/kg was administered every 2 weeks. None of the patients had previously received immunotherapy. 10 of 34 (29.4%) patients in cohort 1 had a response in CNS. Interestingly, 7 patients had discordance between CNS and systemic responses (4 with PD in brain and PR in body, and 3 with PR in brain and PD in body). Intracranial PFS among patients with BM response or stable disease was 10.7 months. Median OS among all patients was 8.9 months, with 31% of patients living at least 2 years. No patients in cohort 2 had a BM response, but 2 of the 5 patients lived past 1 year. Treatment was well-tolerated, with no neurologic adverse events (AEs)  $>$  grade 1 related to treatment, although 2 patients developed grade 3 pneumonitis related to pembrolizumab<sup>[42]</sup>. Although large prospective studies are lacking, taken together, it appears that a PD1 inhibitor can have activity in brain and may be a safe and active treatment option for patients with small, asymptomatic BM in NSCLC patients.

The role of immunotherapy has been more extensively studied in melanoma patients with BM. Ipilimumab, a CTLA4 inhibitor, achieved intracranial disease control in 12/51 (24%) patients in patients with asymptomatic small BM without steroid use<sup>[43]</sup>. More recently, two phase 2 studies combining nivolumab and ipilimumab were investigated in the same patient population. The Checkmate 204 study comprised short-term follow-up of 75 asymptomatic patients. The ICRR was 56% with 20% of patients experiencing CR<sup>[44]</sup>. In an Australian study, 35 and 25 patients with asymptomatic BM and no previous immunotherapy were randomized to cohort A (nivolumab plus ipilimumab) and B (nivolumab only), respectively. 16 patients in whom local therapy had failed or who had neurological symptoms or leptomeningeal disease were enrolled in cohort C<sup>[45]</sup>. The ICRR, CR for cohort A was 46% and 17%, respectively. The median intracranial PFS was not reached at data cutoff. Of note, for treatment-naïve patients, the ICRR was 56%. By contrast, the cohort B of 25 patients receiving nivolumab only had ICRR of 20% and CR of 12%, respectively with median intracranial PFS of 2.5 months<sup>[45]</sup>. In contrast to cohort A and B, only one of 16 patients in cohort C had intracranial response (6%) and this patient had BRAF wild type, no leptomeningeal disease and neurological symptoms only<sup>[45]</sup>. As expected, more treatment-related adverse events (AEs) happened in cohort A patients who received combination. Three patients (9%) in cohort A and one patient in cohort C (6%) developed peripheral neuropathy. Grade 3 AEs occurred in 54% of cohort A, 16% of cohort B and 13% of cohort C and the most common AEs were diarrhea or colitis, and hepatitis. Three patients in cohort A experienced grade 4 hepatitis, pulmonary edema or hypophysitis. One patient in cohort B and one patient in cohort C experience severe headache due to cerebral edema. No death occurred because of study treatment<sup>[45]</sup>. No CNS demyelination occurred, although it was reported previously in a patient treated with nivolumab after progressing on ipilimumab<sup>[46]</sup>. These results suggest that combination immunotherapy with ipilimumab and nivolumab may represent a valuable option in patients with asymptomatic melanoma brain metastases, long-term follow-up with OS data is eagerly awaited<sup>[45]</sup>.

## FUTURE PERSPECTIVE

In general, the response of systemic (extra-cranial) disease correlates well with that of intracranial BM with immunotherapy although discordance has been reported<sup>[42,45]</sup>. Using the available biomarkers, we might be able to tease out the patients with asymptomatic small BM who might respond well to immunotherapy or an immunotherapy combination. At this time, there are no data demonstrating the use of combination immu-



notherapy in NSCLC with BM. However, as aforementioned, the Checkmate 227 study exhibited great activity of nivolumab-ipilimumab combination against NSCLC with high TMB<sup>[28]</sup>. Extrapolating from melanoma studies, it is conceivable that the same combination might be able to confer a much higher and more durable response in NSCLC with BM and high TMB. In the Keynote 189 trial using platinum/pemetrexed/pembrolizumab, the hazard ratio in the subgroup of 108 patients with BM was impressive 0.36, suggesting chemo-immunotherapy could represent an effective option for this group, although PDL1 status was unknown<sup>[26]</sup>. For the PD-L1  $\geq 50\%$  subgroup, the RR was over 60%, with a disease control rate almost up to 90%<sup>[26]</sup>. This treatment combination might also provide a great ICRR and durable IC PFS given that both chemotherapy and immunotherapy have been shown to be effective against BM in NSCLC. Furthermore, how to incorporate and/or sequence SRS in the context of immunotherapy remains unanswered. Prospective studies are needed to address these burning questions, and future adoption of immunotherapy or immunotherapy combinations as the first-line therapy in the appropriate patient population with BM may change our current clinical practice and the use of WBRT or SRT.

## DECLARATIONS

### Authors' contributions

Design: Niu J

Literature research, data analysis, manuscript writing and editing: Niu J, Zhou J, Lindebak S, Mahmoud F

Manuscript revision: Niu J

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Not applicable.

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Original Article

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# *In silico* design of novel gold-phosphate containing compounds as selective inhibitors of cathepsin B in neuroinflammation

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## ABSTRACT

**Aim:** Alzheimer's disease is characterized by pathological protein aggregates and microglia-driven chronic neuroinflammation. Cathepsin B has been proposed as the potential target for inhibiting adverse activation of microglia and slowing down this neurodegenerative disease. Currently available inhibitors of cathepsin B enzymatic activity are non-selective; therefore, the design and synthesis of novel specific inhibitors could facilitate the development of a new class of anti-Alzheimer medications targeting the neuroinflammatory component of this disease.

**Methods:** We describe molecular design strategies, which were used to create specific cathepsin B inhibitors based on the structure of the gold-containing drug auranofin (Ridaura), and its covalent binding to the cysteine residue of the active site of cathepsins.

**Results:** This *in silico* study investigated the structure-activity relationship of a series of newly designed derivatives of auranofin with regard to their cathepsin B inhibitory activity. An exhaustive molecular screening model was designed and validated by using a set of known cathepsin B inhibitors. Its validity was further tested during



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the preliminary stage of the biological screening of newly designed inhibitors. Based on the structure-function relationships discovered by recording the empirical score values generated for the screening model, a series of subsequent *in silico* predictions of compound inhibitory activity were generated, which led to new structures with increased inhibitory activity and selectivity towards cathepsin B.

**Conclusion:** The described molecular modeling strategy could be employed to design novel inhibitors of cathepsin B enzymatic activity, which could be used to slow down neuroinflammation in neurodegenerative disorders including Alzheimer's disease.

**Keywords:** Anti-inflammatory drugs, auranofin, cathepsins, drug design, fragment-based docking, neuroinflammation, microglia

## INTRODUCTION

Alzheimer's disease is the most common cause of dementia. It is characterized by abnormal brain protein deposits including amyloid  $\beta$  (A $\beta$ )-containing plaques and neurofibrillary tangles. In addition, chronic neuroinflammation, driven by adverse activation of non-neuronal microglial cells, is believed to contribute to the pathogenesis of this neurodegenerative disease. Microglial immune functions can be regulated by various cathepsin enzymes, many of which are the components of lysosomes<sup>[1,2]</sup>. Cathepsins belong to the papain superfamily and are synthesized as inactive pro-enzymes<sup>[3]</sup>. Cathepsin B, a cysteine protease, is expressed and can be secreted by activated microglia<sup>[4-7]</sup>. Cathepsin B has been proposed as a potential therapeutic target to reduce neuroinflammation in Alzheimer's disease<sup>[8]</sup> based on observations showing this protein upregulated in brain tissues, serum and cerebrospinal fluid of Alzheimer's patients<sup>[9-13]</sup>. Such high levels of cathepsin B also correlate with decreased cognition, which could be caused by adverse microglia activation<sup>[11,14]</sup>.

A series of pre-clinical studies support using cathepsin B inhibitors to slow the progression of Alzheimer's disease. Reduced A $\beta$  plaque load and improved memory were reported in a transgenic Alzheimer mice model after deleting the cathepsin B gene<sup>[15]</sup>. *In vitro* studies showed that cathepsin B inhibitors downregulated pro-inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-18<sup>[16-18]</sup>. Cathepsin B has been linked to specific microglial functions including their secretion of cytokines, neurotoxicity, and A $\beta$  degradation<sup>[5,17,19]</sup>. Currently available cathepsin B inhibitors are not specific since they inhibit enzymatic activity of other cysteine proteases including calpains, as well as cathepsins S and L<sup>[3,20-23]</sup>. Therefore, novel highly selective cathepsin B inhibitors should be developed and tested for their ability to ameliorate neuroinflammation in neurodegenerative diseases.

Cathepsin B enzymatic activity is inhibited by alpha-macroglobulin from the cystatin family of inhibitors of papain-like cysteine peptidases, and by representatives of the equistatin family<sup>[24]</sup>. There are three additional groups of naturally occurring cathepsin B inhibitors: the aziridinyl peptides, peptide epoxysuccinyls, and peptide aldehydes<sup>[25,26]</sup>. Known synthetic cathepsin B inhibitors can be divided into groups of compounds, which contain either flavonoids, cyclic sulfates, or nitriles<sup>[27,28]</sup>. Cathepsins B and K are inhibited with reasonable potency by gold(I)-based compounds such as auranofin (Ridaura), which is clinically used as an anti-rheumatic agent, and its analogs<sup>[29]</sup>. Structure-activity relationship (SAR) studies revealed that replacement of ethyl substituent with a voluminous aryl substituent in auranofin, which is a clinically approved triethylphosphine (P $\text{Et}_3$ ) gold-containing drug, significantly increased its anti-inflammatory activity<sup>[30]</sup>. A triphenylphosphine gold compound was shown to be a more effective cathepsin B inhibitor ( $\text{IC}_{50}$  = 337 nmol/L) than its parent compound auranofin ( $\text{IC}_{50}$  > 250  $\mu\text{mol/L}$ )<sup>[30]</sup>. Further studies confirmed that compounds with more than one aryl group (e.g., triarylphosphines) were much stronger cathepsin B inhibitors than triethylphosphine Au(I)-containing derivatives<sup>[31]</sup>.

In this study, we describe an *in silico* model developed for identification of pharmacophores capable of increasing the biological activity of Au(I)-based drug-like compounds. Previously, it was shown that changes in steric and electronic properties of phosphine derivatives led to increased affinity of these compounds toward cathepsin B<sup>[31]</sup>. The aim of the present study was to develop and test novel structural modifications of cathepsin B inhibitors, which could be used for synthesis of new, potentially more effective anti-neuro-inflammatory drugs. To achieve this goal, we first developed an *in silico* docking model of the cathepsin B enzymatic pocket. Subsequently, a series of novel cathepsin B inhibitors were designed and synthesized, based on their calculated binding affinity to the enzymatic pocket and docking scores. An *in vitro* testing of selected compounds as possible cathepsin B inhibitors was also performed.

## METHODS

### *In silico* modeling

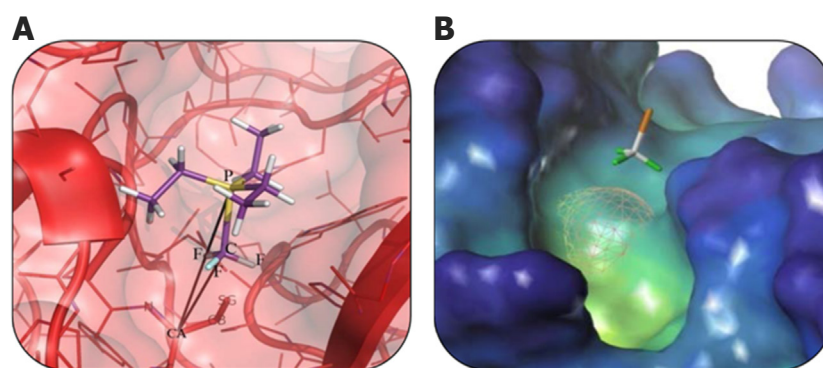
All manipulations with protein-ligand structures and generation of structural models of cathepsin-ligand complexes were performed with Sybyl-X software (Tripos Inc., St. Louis, MO, USA). The three-dimensional structure of the triethylphosphine was generated by the CONCORD version 3.0 software (CONCORD, St Louis, MO, USA). Ligand topologies for molecular dynamics (MD) studies were calculated using the ante-chamber module of AmberTools version 12<sup>[32]</sup>. The protein structures of cathepsins B and K (Protein Data Bank Identifiers (PDB IDs): 1HUC and 2ATO) were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB, [www.rcsb.org](http://www.rcsb.org))<sup>[33]</sup>. Structures of the complexes formed by cathepsin B interacting with triethylphosphine, as well as cathepsin K with myochrysine (Au-thiomalate), were modeled in the experiment. The subsequent steps of the relaxation strategy for the structural optimization of active site geometry were carried out with Gromacs (version 4.5) software with implementation of Amber99 force field<sup>[34]</sup>.

Due to the absence of defined atom-type and coordination bonding parameters for Au in both MD and docking software, we utilized fragment-based docking methods for the model development. Replacement of the Au atom with a CF<sub>3</sub> group, which has four heavy atoms, allowed us to preserve both geometry and distances between the ligand and C-alpha of the catalytic residue Cys29 (C29) in the cathepsin B molecule [Figure 1]. As a result of this manipulation, a “joint-like” metal-based binding of Au was replaced with the “anchor-like” binding mode of CF<sub>3</sub>. Additional mutation of the catalytic cysteine (C29) to alanine (C29A) provided more flexibility for the CF<sub>3</sub>-containing ligands and avoided artificial interaction between the sulfur atom of C29 and the CF<sub>3</sub> group.

In order to remove any unfavorable steric clashes between the proteases and the inhibitor molecules, 1000 steps of steepest descent and 5000 steps of conjugate-gradient energy minimization were employed. The complex between cathepsin B and triethylphosphine was solvated with a 12 Å radius water box centered at the C29A. A center of mass (COM) pulling mode was applied during all steps of MD calculations to improve the geometry of the P-CF<sub>3</sub>-C-alpha (CA, of alanine) bridge. The optimized and equilibrated system was used as the starting configuration for MD simulations spanning 500 ps. Such fast molecular dynamics was enough to stabilize the group of constrained P-CF<sub>3</sub>-CA atoms. Average root-mean-square deviation (RMSD) values for the whole structure, including the active site and ligand atoms, were calculated from each of the five picoseconds frames of the simulation. RMSD values, together with the structure alignment, confirmed that conformation of the ligand and the active site of protein had not changed dramatically and were considered stable enough to continue modeling.

For the docking procedure, a specific binding site representation (namely Protomol) was generated based on the relaxed protein-ligand complex. Protomol is an interaction map, implemented in the Surflex-Dock module, which is based on the probing method used<sup>[35]</sup>. Protomol model is very sensitive to input parameters; therefore, to avoid mistakes in predictions, it was parameterized based on scores obtained through validation docking procedures. To keep the amino acid environment flexible during each docking run, the most





**Figure 1.** A schematic representation of the mutual orientation and disposition of P-CF<sub>3</sub> part of triethylphosphine and C-alpha (CA) atom of the Cys29 residue of cathepsin B (A). Active site of cathepsin B with the CF<sub>3</sub> group (F atoms are colored green) (B). Cys29 is replaced with Ala29 (the surface of Cys29 is shown in mesh)

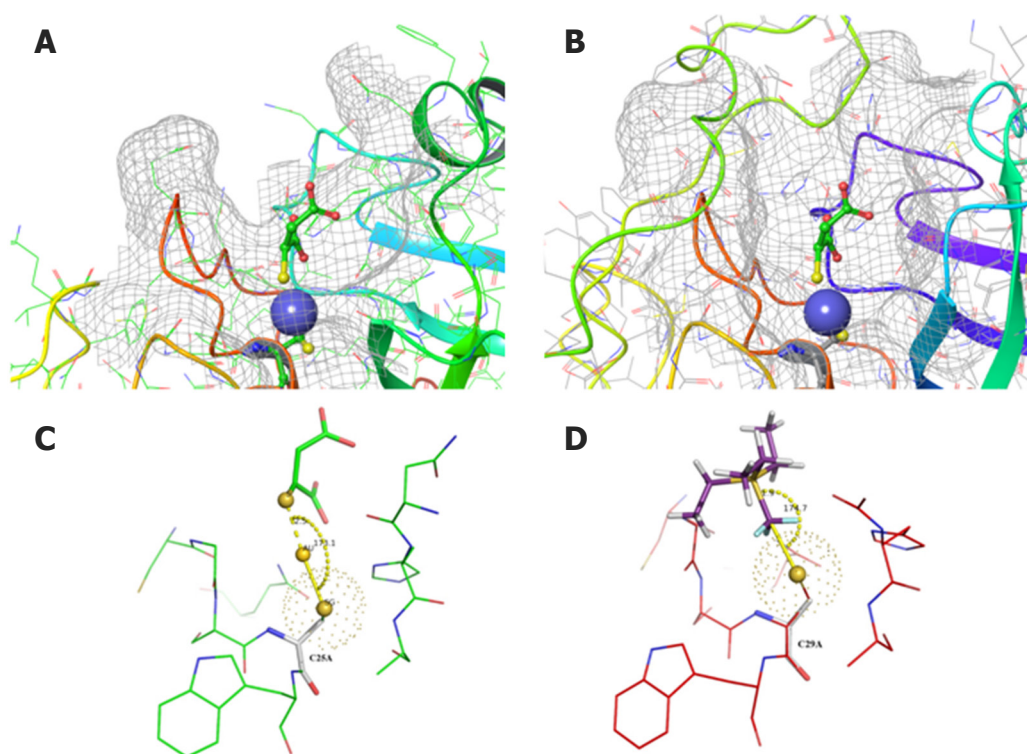
reliable positions of the reference PEt<sub>3</sub> were retained with the number of starting conformations per ligand higher than ten. The new compounds were designed based on the shape of the binding pocket and its hydrophobic/hydrophilic areas.

A set of reference compounds was compiled from publicly available structures and contained the triethylphosphine molecule and its modifications. Structures of these reference compounds and their corresponding IC<sub>50</sub> values as cathepsin B inhibitors have been previously published<sup>[36]</sup>. Predictions from the docking studies were made based on correlations between docking scores and known enzymatic activity of the reference compounds. Surflex-Dock scores (total score expressed in:  $-\log_{10}(K_d)$  units) represented the binding affinities. Four different calculated parameters were used as the most important indicators in docking analysis: “total score”, “crash value” (the degree of inappropriate penetration by the ligand into the protein; values close to zero are favorable), “internal ligand strain” and “complex absolute energy”. In addition, the root-mean-square (RMS) distance between the docked ligand and the reported fragment RMSD (FragRMSD) was calculated to provide the correct orientation of the ligand in the binding site.

### ***In vitro* testing**

Cathepsin B enzymatic activity was measured as described by Hulkower *et al.*<sup>[37]</sup> (2000) with modifications. A fluorometric assay was used in which cathepsin B (from bovine spleen) cleaved its substrate, Z-Arg-Arg-AMC (both from Sigma Aldrich, Oakville, ON, Canada), causing the cleaved product to fluoresce. The reaction was performed in Hanks' balanced salt solution containing 0.6 mmol/L CaCl<sub>2</sub>, 0.6 mmol/L MgCl<sub>2</sub>, 2 mmol/L L-cysteine, and 25 mmol/L PIPES, pH 7.0. The assay was performed in a 96-well plate, and the POLARstar Omega plate reader (BMG Labtech, Durham, NC, USA) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm was used to measure the velocity of the reaction (relative fluorescence units per min) at 37 °C. Au-containing substances were dissolved in dimethyl sulfoxide (DMSO) and added to the reaction mixture at 10 nM to 500 μmol/L concentration range. The final concentration of the solvent in the reaction mixture did not exceed 0.5%. The solutions containing cathepsin B (200 μg/mL) and the inhibitors were allowed to incubate for 30 min at 37 °C. Subsequently Z-Arg-Arg-AMC (from a 600 μmol/L stock solution) was added to the wells to reach the final concentration of 30 μmol/L in a total well volume of 180 μL. The 96-well plate was then positioned into the plate reader and fluorescence measurements from each well were recorded. The plate reader was set to acquire 64 measurements over a 52-min time frame. The maximum slope values of the samples containing inhibitors were calculated as percentages of maximum slope values of the control samples containing DMSO vehicle solution only, and IC<sub>50</sub> values for each inhibitor determined.





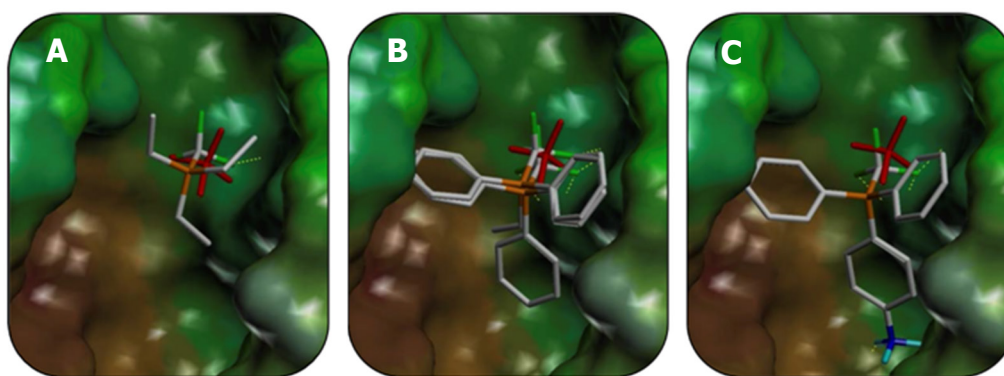
**Figure 2.** Cathepsin K (A) with co-crystallized and cathepsin B (B) with superimposed myochrysin (Au-thiomalate) are represented in mesh surface and colored ribbon style. Au atom is shown as a blue sphere. A secondary structure color scheme (suggests different colors for different secondary structures) was used for ribbon representation. A schematic representation of ligand binding mode and the geometry of the Au atom and the sulfur bridge in cathepsins K (C) and B (D). Ionic bond length and angle between the terminal sulfur of Cys25 of cathepsin K are indicated. The same view of CF<sub>3</sub>-substituted triethylphosphine in the active site of cathepsin B with mutated Cys29 to Ala29 is shown in (D)

## RESULTS

Proper selections of binding sites, together with a good understanding of the mechanism of enzymatic activity of cathepsins, are the key factors for designing effective new inhibitors; however, currently available inhibitors of cathepsin B are only 2- to 8-fold more selective towards this enzyme compared to two other cathepsins S or L<sup>[3,22]</sup>. It was previously shown that cathepsin B is reversibly and competitively inhibited by linear Au(I)-containing complexes<sup>[29]</sup>. A hit-to-lead optimization of the organic ligands used to create new auranofin analogs, particularly the phosphine ligands, could significantly increase the potency of these compounds as cathepsin B inhibitors. Phosphine ligand interaction with cathepsin B was modeled based on the myochrysin (Au-thiomalate) binding to cathepsin K<sup>[31]</sup>. The crystal structure of the 339 amino acid-long human liver cathepsin B has been published at a 1.9 Å resolution (PDB ID: 1GYM)<sup>[38]</sup>.

Access to the active site of cathepsin B is provided by an 18 amino acid long insertion (Pro107Asp124), termed the occluding loop, which possesses two His residues for binding of the carboxyl group of the substrate. Three-dimensional alignment of the crystal structures of cathepsins B, S, K and L demonstrated a considerable degree of homology, especially in the region of substrate binding. Our molecular modeling identified that the potential binding site on the surface of cathepsins is represented by a long hydrophobic pocket, which is necessary for the peptide binding and excision. Figure 2 shows that the structures of cathepsins K and B are very similar, including their cleavage sites containing several “hot-spot” amino acids conserved among all types of cathepsins, along with Cys29, which forms a coordination bond with PEt<sub>3</sub>.

Despite the great variety of protein modeling tools and techniques available, there is no simple approach to study interactions between Au-containing compounds and proteins without the time-consuming quan-



**Figure 3.** Orientations of the most potent reference enzymatic activity inhibitors  $P(CH_2CH_3)_3$  (A),  $P(CH_2CH_3)(C_6H_5)_2$  (B), and  $P(C_6H_5)_3$ ,  $P(C_6H_4NH_3^+)(C_6H_5)_2$  (C) in the active site of cathepsin B

tum mechanics (QM) calculations. The absence of Au or any cognate element and its bond parameters in most force field and docking protocols compelled us to implement several alternative techniques, known as steered molecular dynamics and fragment-based docking approach, as suitable models for docking and subsequent lead optimization. A structure preparation tool from SybylX suite was used to model the complex between cathepsin B and Au-containing ligand. A crystal structure of cathepsin B (PDB ID: 1HUC) was superimposed on the cathepsin K structure (PDB ID: 2ATO) and coordinates of co-crystallized myochrysin (Au-thiomalate) and cathepsin B were saved for subsequent analyses. The highly conserved cysteine, histidine and asparagine residues from the active site of cathepsins B and K were used for structure superposition.

The measured essentially important linear angle between the atoms in the S-Au-S triad (sulfur of C25, Au(I) and sulfur of thiomalate) of the complex between cathepsin K and Au-thiomalate was  $173.1^\circ$ . Subsequently, the gold atom in Au-thiomalate was replaced with a  $CF_3$  group.  $CF_3$  is a directional and rigid group, which has a binding mode similar to the gold atom. The  $CF_3$  possesses four tetrahedral heavy atoms, which are necessary for accurate geometric constraints as well as the bond-like rotation during molecular dynamics and docking. In addition, the Cys25 residue in the enzymatic pocket of cathepsins B and K was replaced with alanine to eliminate a steric clash and electrostatic interactions with the  $CF_3$  group of thiomalate. In order to validate the docking procedure, a model of cathepsin K was developed based on its complex with  $CF_3$ -containing Au-thiomalate. Subsequently,  $CF_3$ -containing Au-thiomalate in this complex was replaced with  $CF_3$ -substituted triethylphosphine.

The fast molecular dynamics simulation, spanning 500 ps, generated parameters of trajectories, which were analyzed and clustered by RMSD to identify the most stable conformation of the complexes. The docking model of cathepsin B was developed based on this structure. Figure 3 shows the resulting modified compound  $CF_3$ -PEt<sub>3</sub> positioned in the mutated (C29A) enzymatic pocket of cathepsin B, with preserved original geometry (position and orientation) of the PEt<sub>3</sub> group. This structure maintained all the distances and co-linear characteristics of the complex between  $CF_3$ -substituted triethylphosphine and cathepsin B. The volume of the cathepsin B active site was defined with a Protomol generation tool.

Next, to provide ligand dislocation similar to that in the  $CF_3$ -substituted complex between triethylphosphine and cathepsin B, we applied both the distance and position constraints. The  $P_{atom}$ - $CF_3$  fragment was assigned as a constraint in Surflex-Dock, to match the correct position and orientation of the ligands in the enzymatic pocket. After several test dockings we chose an appropriate value for “cpen” function (penalty for deviating from fragment), which determines how closely compounds could be positioned to the source coordinates of the template P- $CF_3$  group of six different  $CF_3$ -substituted auranofin derivatives. Reference compounds

**Table 1. Scoring values of ligands docked in the active site of cathepsin B**

Sample	Total score	Crash score	Internal strain <sup>†</sup>	Complex energy	FragRMSD <sup>#</sup>	IC <sub>50</sub> (μmol/L)
CF <sub>3</sub> P(CH <sub>2</sub> CH <sub>3</sub> ) <sub>3</sub> Triethylphosphine	1.325	-0.211	0.073	482.7	0.745	~250
CF <sub>3</sub> P(C <sub>6</sub> H <sub>5</sub> ) <sub>2</sub> (CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub> )	3.602	-0.600	0.252	626.0	0.970	~64
CF <sub>3</sub> P(CH <sub>2</sub> CH <sub>3</sub> )(C <sub>6</sub> H <sub>5</sub> ) <sub>2</sub>	4.051	-0.952	5.485	560.0	0.737	18.0
*Cmpd 4	4.201	-1.505	2.500	658.5	0.97	8.67
Cmpd 2	3.991	-0.003	2.449	594.1	1.123	1.00
Cmpd 5	6.560	-1.080	2.201	660.0	0.85	0.71
Cmpd 3	4.059	-0.919	0.918	624.4	0.997	0.46
Cmpd 1	4.747	-0.937	0.157	575.0	0.617	0.34
CF <sub>3</sub> P(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub>	4.353	-1.150	2.001	607.6	0.840	0.33
CF <sub>3</sub> P(C <sub>6</sub> H <sub>4</sub> -C <sub>6</sub> H <sub>5</sub> )(C <sub>6</sub> H <sub>5</sub> ) <sub>2</sub>	4.401	-1.301	0.452	613.1	0.861	0.29
CF <sub>3</sub> P(C <sub>6</sub> H <sub>4</sub> NH <sub>3</sub> <sup>+</sup> )(C <sub>6</sub> H <sub>5</sub> ) <sub>2</sub>	6.940	-1.130	3.157	731.2	1.002	0.20
Cmpd 6	6.938	-1.640	0.4503	703.6	0.80	0.17

\*Cmpd 1-6: the newly synthesized compounds 1-6; <sup>†</sup>Internal strain: nominal ligand strain relative to the nearby local minimum in units of pKd;

<sup>#</sup>fragment root-mean-square deviation

from an open source database and previous publications were selected for validation of the MD model<sup>[36,39]</sup>. Through the design of novel compounds, we aimed to improve their cathepsin B binding affinities. The three-dimensional structure of the new compounds was generated by the CONCORD (see Methods section). We chose a triphenylphosphine (PPh<sub>3</sub>) as the initial structure for design, since previous studies<sup>[29]</sup> confirmed its significant *in vitro* activity as an inhibitor of cathepsin B enzymatic activity. The validation step of the fragment-based docking defined optimal software settings, which provided the best correlation between the enzymatic activity inhibition data and the docking score [Table 1].

Table 1 lists a set of compounds with known IC<sub>50</sub> values in cathepsin B enzymatic assays. This table also includes the previously reported compound triethylphosphine (P(CH<sub>2</sub>CH<sub>3</sub>)<sub>3</sub>) and its analogs with substituted methyl groups on phenyl rings<sup>[31]</sup>. Table 1 likewise includes examples of newly synthesized compounds (Cmpd 1-6). These novel derivatives were created by replacing the triethylphosphine ligand of the auranofin molecule with other phosphines carrying various aromatic and heteroaromatic substituents. Table 1 is divided into seven columns and the most practical parameter for the docking analysis outcome is the summarized score (total score), which is an empirical affinity value developed to predict the potency of the inhibitors. This value is calculated based on the other parameters shown in this table. The upper part of the table illustrates correlations between scores obtained from *in silico* modeling and *in vitro* testing described in published studies and patents<sup>[31,36,40]</sup>. The lower part of the table shows predicted affinities and the measured cathepsin B inhibitor activities for the newly synthesized compounds. The validation step was complete after confirmation of a relationship between the calculated and biochemically determined values for known inhibitors. These two parameters are listed in Table 1 and compounds are sorted in order of ascending IC<sub>50</sub> values in cathepsin B enzymatic activity assay, which correlate inversely (negative correlation coefficient of -0.71) with the calculated total score values.

In addition, we repeated all docking procedures with cathepsin K, exactly as they were described for cathepsin B above, by applying match constraining to CF<sub>3</sub>. In this way, we compared the results of our MD modeling for both proteins, which showed different docking scores for the interactions between the substituted triethylphosphine derivatives and cathepsin K [Table 2]. These data indicate a certain degree of selectivity towards a specific cathepsin isoform for these compounds.

Three-dimensional alignments of docked reference inhibitors in the active site of cathepsin B are presented in Figure 3. RMSD values for the compounds used to estimate the three-dimensional matching of the reference arylphosphine derivatives to substituted triethylphosphine are listed in Table 1. After the initial lead

**Table 2. Scoring values of ligands docked in the active site of cathepsin K**

Sample	Total score	Crash score	Internal strain	Complex energy	Fragment RMSD*
CF <sub>3</sub> P(CH <sub>2</sub> CH <sub>3</sub> ) <sub>3</sub> triethylphosphine	1.3065	-0.4537	0.0682	369.7	0.694
CF <sub>3</sub> Au-thiomalate	1.984	-0.3104	0.034	317.7	0.705
CF <sub>3</sub> P(CH <sub>2</sub> CH <sub>3</sub> )(C <sub>6</sub> H <sub>5</sub> ) <sub>2</sub>	2.4274	-0.4531	0.0663	390.3	0.528
CF <sub>3</sub> P(C <sub>6</sub> H <sub>5</sub> ) <sub>3</sub>	3.4247	-1.4016	4.5	451.1	0.901

\*Fragment root-mean-square deviation

compound optimization and docking, a limited set of seven substituted phosphines were synthesized from a total of 15 designed structures and used for *in vitro* studies. The best way of comparing the overall calculated binding affinities of ligands is a comparison of their total score values. However, all other scoring functions can be useful in specific cases. For example, as can be seen from Table 1, the best experimental and docking results were obtained for P(C<sub>6</sub>H<sub>4</sub>NH<sub>3</sub><sup>+</sup>)(C<sub>6</sub>H<sub>5</sub>)<sub>2</sub> compound. It is evident that high total scores and low internal strain scores are preferable for the newly developed compounds. Therefore, these parameters were used for the further design of the improved novel drug-like compounds. Further optimization and improvement of each drug-like compound were based on their spatial location in the enzymatic pocket of cathepsin B and compliance to the physical features of the active site, including anchoring with the CF<sub>3</sub> group and localization of phosphine groups in the hydrophobic/hydrophilic regions of the enzymatic pocket.

## DISCUSSION

Our data show that the described docking model is a practical tool for identification and optimization of novel compounds, which have been designed based on their phosphine core structure. We also focused our attention on the shape features of the cathepsin B binding site and achieved a good trend in selectivity of the inhibitors towards this enzyme, by avoiding their binding to cathepsin K. The inverse correlation (-0.71) between docking scores of compounds and their IC<sub>50</sub> values as well as significant selectivity towards cathepsin B and not cathepsin K [Tables 1 and 2] supported subsequent docking analysis and selection of compounds for *in vitro* testing. Thus, by using the molecular modeling described in this study we were able to design and create structurally novel derivatives of the clinically available anti-rheumatic drug auranofin, which inhibited the enzymatic activity of cathepsin B more effectively than their parent drug. It has been established that the clinical anti-inflammatory activity of auranofin depends on its ability to affect multiple cellular and molecular targets<sup>[41]</sup>. Even though clinical effectiveness of auranofin in neurodegenerative disorders has not been studied, its anti-inflammatory activity may be beneficial for slowing down neuroinflammation accompanying such pathologies as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis<sup>[42]</sup>. Optimizing the activity and selectivity of auranofin molecule as an inhibitor of cathepsin B through structural modifications may lead to additional benefits of such novel compounds in Alzheimer's disease in particular<sup>[43]</sup>. Further *in vivo* studies will be required to determine the pharmacokinetics and pharmacodynamics of these novel derivatives of auranofin, as well as their clinical suitability as anti-neuroinflammatory drugs and cathepsin B inhibitors.

## DECLARATIONS

### Authors' contributions

Conceived the study and wrote the manuscript: Raevsky AV, Sharifi M, Pinchuk V, Klegeris A  
Conducted modeling experiments and analyzed the data: Raevsky AV, Sharifi M

### Availability of data and materials

Data in this study were obtained by experimentation and through *in silico* modeling, and are original. All primary data used to construct the figures and summary tables are available by contacting the authors of this study.



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### Conflicts of interest

The authors declare no conflict of interest. M. Sharifi declares the view presented in this article are those of the author and do not reflect those of the US Food and Drug Administration. No official endorsement is intended nor should be inferred.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Original Article

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# The novel-molecule T11TS facilitated arousal of glioma-mediated dormancy of bone-marrow hematopoietic stem-cells

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## Abstract

**Aim:** T11TS, a potent anti-gliomagenic glycoprotein, stimulates both peripheral and intracranial immune response. The status of bone marrow hematopoietic stem cells (BMHSCs), the cradle of regeneration of all blood cells, during gliomagenic global immune devastations has not yet been investigated. Therefore, we aimed to delineate the effects of T11TS on immature and mature compartments of hematopoietic machinery.

**Methods:** Flowcytometric analysis of cultured BMHSCs was evaluated for assessing the expression pattern of early hematopoietic stem cells (HSCs) markers such as CD34<sup>+</sup>, Sca-1<sup>+</sup>, c-kit<sup>+</sup> and also Angiopoietin-1 and Tie-2 both in normal, glioma, and in T11TS treated glioma-bearing animals. Immunofluorescence imaging and western blot analyses of BMHSCs were also carried out.

**Results:** There was significant downregulation of HSCs-markers CD34<sup>+</sup>, Sca-1<sup>+</sup>, c-kit<sup>+</sup> in ethyl nitrosourea-induced glioma-bearing animals followed by an increase in the expression level of Ang-1 and Tie-2 that determines the quiescence and self-renewability of stem cells. T11TS administration reversed the gliomagenic transformation of expression of the above mentioned markers. The results flowcytometric-analysis was also well corroborated with immunofluorescence imaging and western blot analysis.



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**Conclusion:** Collectively, the above experimental evidence hints towards gliomagenic maneuver of receptor expression of HSCs to derange the systemic immunity and T11TS mediated manipulation towards revival/rejuvenation of the same.

**Keywords:** Glioma, immunosuppression, T11TS, immunotherapy, hematopoietic stem cells, immunophenotyping, immunomodulation

## INTRODUCTION

Systemic depression of cellular immunity and subsequent global immune suppression is the classical poor prognostic fact of gliomagenesis<sup>[1]</sup>. Debilitating gliomas evade the host immune surveillance either by adopting immune-editing strategies such as impairment of antigen presenting machinery<sup>[2]</sup>, activation of negative co-stimulator signals for example cytotoxic T-lymphocyte-associated protein 4 (CTLA4), B7 homolog 1/programmed death 1 (PD1) and Programmed death-ligand 1 (PDL1)<sup>[3-5]</sup> and recruitment of pro-apoptotic pathway [e.g., Fas receptor, Fas ligand (FasL)]<sup>[6]</sup>, expansion of regulatory T cell population<sup>[7]</sup> and down regulation or absence of tumor specific antigen<sup>[8]</sup> or by secreting cytokines such as interleukin 10 (IL-10), interleukin 6 (IL-6), transforming growth factor beta 1 (TGF- $\beta$ ), prostaglandin E2 (PGE2), and various gangliosides which have been implicated for their direct immune suppressive role to diminish the antitumor immune response during glioma progression<sup>[9-11]</sup>.

Interestingly, gliomagenic generation of potent immune suppressors in addition acts as intense inhibitory regulators of hematopoiesis by modulating the function of bone marrow hematopoietic stem cells (BMHSCs)<sup>[12,13]</sup>. TGF- $\beta$ , a key negative regulator of hematopoiesis not only suppresses *in vitro* proliferation of both progenitors and mature stem cells but also induces quiescent state of functional hematopoietic stem cells (HSCs)<sup>[14,15]</sup>. Malignant brain tumor derived gangliosides inhibited hematopoiesis at different stages of differentiation and resulted in bone marrow hypoplasia<sup>[16,17]</sup>. PGE2 regulates hematopoiesis in a dose and time-dependent manner and modulates hematopoiesis as negative and positive feedback control<sup>[18,19]</sup>. The immunosuppressive, IL-10 is found to contribute its inhibitory impact in hematolymphopoiesis during various pathological conditions<sup>[20,21]</sup>.

Viewing HSCs as the foundation for the immune response<sup>[22]</sup> we hypothesize that global immune suppression during glioma progression might have an important deleterious bearing on synchronized internal signaling network of hematopoietic machinery that regulates HSCs homeostasis, proliferation, and migration. However, there is an iota of literature about the status of bone marrow-derived HSCs during glioma condition and/or immunotherapeutic modulation of HSCs. There has been little success in preventing destructive nature of glioma either by modulating glioma stem cells or by using genetically engineered stem cells in glioma therapy<sup>[23,24]</sup>.

HSCs are delineated by their propensity for self-renewal and differentiation into entire committed hematopoietic lineages. With few exceptions, proper functioning of early hematopoietic stem and progenitor cells are dependent on c-kit, stem cell antigen-1 (Sca-1) and CD34 mediated signalling system for their self-renewal, proliferation, and survival<sup>[25-27]</sup>. On the other hand, Angiopoietin-1 (Ang-1) and tyrosine-protein kinase receptor (Tie-2), the two important cell surface markers maintain homeostasis of HSCs by regulating molecular crosstalk between HSCs and bone marrow niche and protect HSCs from pathological conditions<sup>[28,29]</sup>.

The novel immunomodulatory glycopeptide, T11 target structure/Sheep-Lymphocyte function-associated antigen-3/CD58 (T11TS/SLFA-3/S-CD58)<sup>[30]</sup>, has been delineated in our lab for its multipotent anticancer activities against N-ethyl-N-nitrosourea (ENU) induced glioma-bearing rat model and also *in vitro* human glioma samples<sup>[31]</sup>. Typically T11TS mediated eradication of glioma is due to the simultaneous rejuvenation of peripheral and intracranial immune system which was profoundly suppressed due to gliomagenic secretion of immunosuppressive cytokines such as IL-10, TGF- $\beta$ , IL-4, and PGE2, *etc.* T11TS imparts immune-potential by activating T-lymphocytes<sup>[32]</sup>, NK cells<sup>[33]</sup>, macrophage (M $\Phi$ )<sup>[34]</sup>, Neutrophils<sup>[30]</sup>

and microglial cells<sup>[35]</sup>. A recent finding by our lab also documented that T11TS favors T cells survival not only by inhibiting glioma mediated apoptogenic death of T cells<sup>[36]</sup> but also by repairing gliomagenic impairment of phosphatidylinositol 3-kinase/AKT (PI3K-AKT) signaling cascades<sup>[37]</sup>. Further study in our lab also deciphered that T11TS immunotherapy, mediate functional activation of T cells by rectifying gliomagenic anti-proliferative action on T-cell by correcting CD2-mediated nuclear factor of activated T-cell calcineurin pathway<sup>[38]</sup>. Some of our previous publications also delineated that T11TS reduces glioma mass simultaneously by accelerating the apoptotic death of brain tumor cells and by decreasing the number of dividing glioma-bearing cells<sup>[39,40]</sup>.

The above immune rejuvenation and induced increased potentiality during T11TS treatment might have an important bearing on the concomitant production of activated immunocompetent cells through the hematopoietic machinery in the bone marrow. The query for such regenerative immunocompetence remains unanswered on the hematopoietic level. Interestingly, in our recent publication, we have elucidated for the first time that T11TS also protects the BMHSCs by inhibiting the premature apoptogenic death by counteracting gliomagenic stimulation of intrinsic, extrinsic apoptogenic pathway and also by inhibiting Granzyme-B mediated apoptotic fate of HSCs within bone marrow milieu<sup>[41]</sup>.

Hence, the present study emphasized on possible modulations of key elements of the early phases of hematopoiesis on bone marrow HSCs during glioma growth and following T11TS therapy. Our finding shows modulatory effects of T11TS therapy towards differentiation, proliferation, activation of HSCs against gliomagenic shock. This entirely new finding not only illuminates the role of HSCs in glioma and also the relevance of T11TS therapy against this dreadful disease, but it may also drive us towards an important new target for basic investigation and, potentially, therapeutic intervention against many more hematological malignancies.

## METHODS

### Animals

Healthy Swiss albino rat pups of both sexes (4.5–6 g) were maintained in our Institutional animal facility as per Institutional Ethical Committee guidelines monitored by Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India regulations<sup>[41]</sup>. Six animals in each group were weaned at 1 month of age and housed in individual cages at 22 °C in a 12 h light/dark cycle. Animals were fed with standard autoclaved food pellets along with water ad libitum.

The experimental animals were grouped into the following 5 groups: (1) age matched normal healthy control [N]; (2) 3–5 days-old neonatal animals intraperitoneally (i.p.) injected with ENU and reared for 5 months (optimal period for glioma development) [ENU]; (3) ENU animals (5 months of age) treated with single dose of (i.p.) T11TS [ET1]; (4) ENU animals treated with 2 doses of (i.p.) T11TS [ET2] at an interval 6 days for each dose; (5) ENU animals treated with 3 doses of (i.p.) T11TS [ET3] at an interval 6 days for each dose.

### Brain tumor induction with ethyl nitrosourea

ENU the engineered alkylating compound is a strong mutagen and is observed to be the most intense of neurocarcinogens engendering brain tumors with architectural and physiological likenesses to typically ensuing neural neoplasms in mankind<sup>[42]</sup>. ENU does not require any metabolic computation for its enactment<sup>[43]</sup>. ENU can enter into brain in spite of an intact blood brain barrier and the alkylated products of the DNA are readily formed within the brain tissue<sup>[44]</sup>.

ENU was freshly prepared by dissolving 10 mg/mL in sterile saline and adjusting the pH to 4.5 with crystalline ascorbic acid. ENU was injected i.p. to newborn rats (3–5 days old) with a dose of 80 mg/kg body weight<sup>[36]</sup>. Maintenance of age-matched control was done by rearing healthy rat pups up to 5 months of age.

### Isolation of T11TS

The glycopeptide T11TS/SLFA-3 was isolated from sheep red blood cell (SRBC) membrane. Briefly, SRBC was trypsinized, and after nonspecific protein precipitation by TCA, was subjected to ion exchange chromatography on a DEAE cellulose column, with a five-chambered gradient system. Finally, elute fraction III was selected as the fraction of choice.

### Administration of T11TS in animals

The first dose of 1 mL of T11TS was administered in rats intraperitoneally (i.p.) from the third elute fraction (EF III), which was followed by a second booster dose on the sixth day and the third booster dose on the day 12, making a dose schedule of 1, 2 and 3 mL to the ET1, ET2 and ET3 animals, respectively<sup>[36,45]</sup>.

### Isolation of HSC from bone marrow

From the long bones (femur, tibia, and fibula) bone marrow was isolated as described previously<sup>[41,46]</sup>. A single cell suspension in an aseptic condition was prepared from the bone marrow. The cells were subjected to centrifugation at 1000 rpm for 20 min on a bi-layered Percoll density gradient namely 1.077 at the bottom and 1.050 at the top. Cells obtained from the top [i.e. low-density compartment (LDC)] and bottom [i.e. high-density compartment (HDC)] layers were collected separately and washed thrice in PBS followed by culturing in RPMI media as described and characterized by Chatterjee *et al.*<sup>[46]</sup>, 2010.

### Short-term stem cell culture

The LDC and HDC cells were cultured and maintained for 5 days in a 75 mm culture dish (Corning, USA) containing 4 mL of RPMI-1640 supplemented with 10% FBS, 100 ng/mL of SCF, 20 ng/mL of IL3 at 37 °C in an atmosphere of 5% CO<sub>2</sub> as described by Mondal *et al.*<sup>[41]</sup>, 2018.

### Effect of T11TS on the phenotypic markers of HSCs

Bone marrow-derived isolated LDC and HDC cells of all groups were taken from 5-day cultures on the 6th day and were subjected to flowcytometric analysis. Percentage of extracellular CD34, Sca-1 and c-kit, Ang-1 and Tie-2 population in LDC and HDC cells were evaluated using primary antibodies against CD34 (BD Biosciences, USA), Sca-1 (Abcam Inc., USA), c-kit, Ang-1 and Tie-2 (Santa Cruz Biotechnology Inc, USA) respectively followed by PE-conjugated anti-rat respective monoclonal antibodies for 30 min as described by Mondal *et al.*<sup>[41]</sup>, 2018 with little modification. FACS Calibur (BD Biosciences) with Cell Quest Pro software was used for acquisition and analysis of percent cellular expression of each protein as quantified. A total of 10,000 events were acquired and analyzed for each sample. Gating was performed with respect to the individual group of the unstained control sample. Values indicated in “section3” are the mean of six individual studies with S.D. calculated oneach mean value.

### Immunofluorescence imaging of cells

Short term cultured HSCs grown overnight on the poly L-lysine coated sterile cover slips at 37 °C were fixed with 4% paraformaldehyde in PBS for 20 min. After blocking, the cells were incubated overnight with anti-primary Ang-1, Tie-2, CD34, and c-kit antibody [1:250 dilutions in PBS with 1% bovine serum albumin (BSA)] at 4 °C. After washing with PBS, CD34 and c-kit tagged cells were incubated with FITC secondary antibody and anti-Ang-1 and Tie-2 tagged cells were incubated with TRITC conjugated secondary antibody (1:500 dilutions in PBS with 1% BSA). The rest of the procedures such as staining of the nucleus with DAPI, visualization, capturing of images and quantification and representation of data were done as described<sup>[41,47]</sup>. Briefly, Nis-Elements D3.00 were used for capturing images and quantification. Each condition was observed in triplicate and six images were taken for each sample. Figures are representative of the group. Results were expressed as mean fluorescence intensity of number total cells positively stained by the desired fluorescence tagged protein out of the total number of cells counted for each experimental group. Figures are representative of the group. As a negative control, primary antibody was omitted.

## Statistical analysis

Data shown are representative of six independent experiments ( $n = 6$ ) and values are expressed as mean  $\pm$  SD unless otherwise stated. For statistical comparison, the one-way analysis of variance was performed followed by application of the Tukey's *post-hoc* test. A  $P$  value  $< 0.05$  was considered to be statistically significant.

## RESULTS

### Bone marrow-derived CD34<sup>+</sup> cell, Sca-1<sup>+</sup> cell and c-kit<sup>+</sup> cell, Ang-1 cell and Tie-2 cell: density specific compartmentalization

During our phenotyping characterization of HSCs from the bone marrow, densitometric centrifugation technique resulted in two well distinct categories of cells. Cells at LDC (1.050) indicate immature HSCs and the other at HDC (1.077) denote mature HSCs<sup>[48]</sup>, which will lead to the generation of the progenitors. The harvested cells were subjected to CD34, Sca-1, c-kit, Ang-1, and Tie-2 positivity in a cell sorter.

### Effect of T11TS on the phenotypic markers CD34 of BMHSCs

Bone marrow-derived CD34<sup>+</sup>-enriched cell population claimed to be one of the most critical markers for HSCs<sup>[49]</sup> and their expression is down-regulated as they differentiate into mature cells<sup>[50]</sup>. Single staining was done for CD34. Flowcytometric analysis [Figure 1A and B] showed a higher enrichment of CD34<sup>+</sup> cells in the LDC level than in the HDC. In normal rats, there was a higher level of expression of CD34 both in the LDC and HDC in comparison to the ENU treated glioma-bearing rat where BMHSCs showed a significantly ( $P < 0.001$ ) decreased level of expression both in the LDC and HDC. However, there was a very significant ( $P < 0.001$ ) increase in expression levels of both in the LDC and HDC in all three dose levels, i.e., ET1, ET2 and ET3 in T11TS treated glioma-bearing rats.

*In situ* immunofluorescence imaging studies [Figure 1C] further confirmed the CD34 FACS findings. In the ENU group, the majority of CD34<sup>+</sup> cells showed a significant ( $P < 0.001$ ) decrease in their expression of fluorescence intensity both in the LDC and (1.692  $\pm$  0.439) in the HDC as compared to the normal group in both compartments. The intensity gradually increased following T11TS therapy in dose dependent manner ET1, ET2 and a significant up-regulation were noted at the third dose, i.e., ET3 in both the LDC and HDC compartments.

### Effect of T11TS on the phenotypic markers Sca-1 of BMHSCs

Sca-1, an important marker of emerging HSCs, regulates the overall developmental program of HSCs towards self-renewal, lineage fate and c-kit expression<sup>[25,51]</sup>.

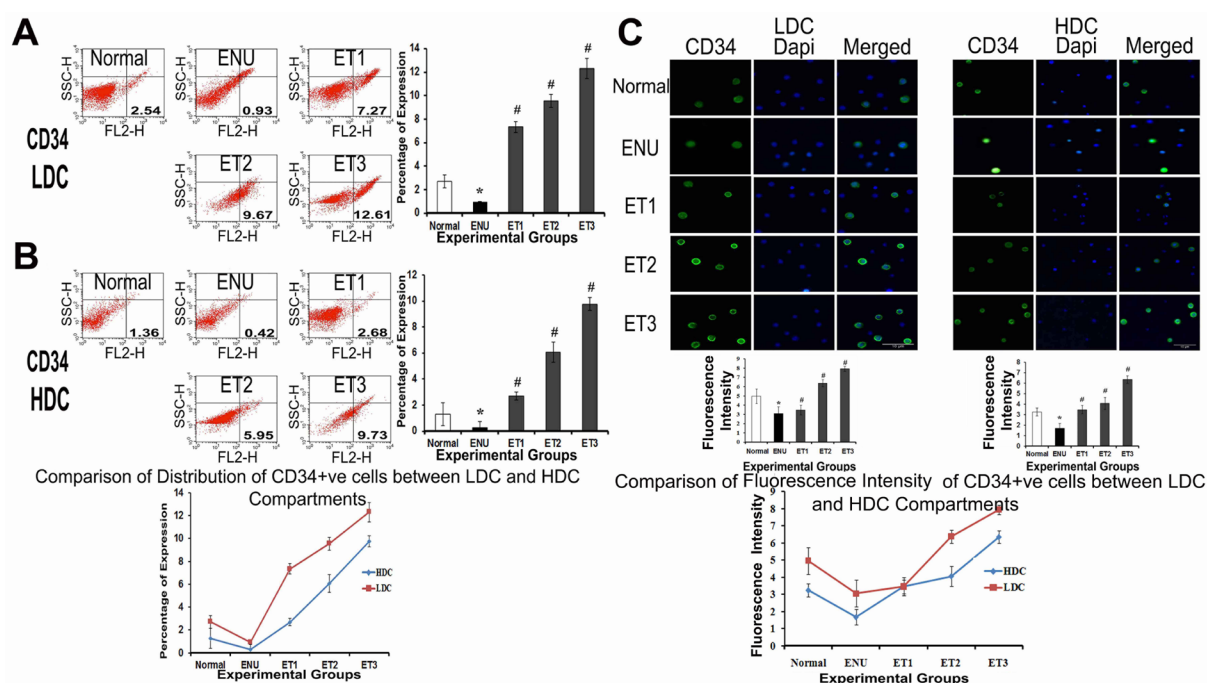
Flowcytometric studies [Figure 2A and B] showed that there was significant down regulation ( $P < 0.001$ ) of Sca-1 expression both in the LDC and HDC (in glioma-bearing rats as compared to the normal groups in both the LDC and HDC. Following T11TS therapy, there was significant up-regulation ( $P < 0.001$ ) of the expression of Sca-1 at all dose levels (ET1, ET2 and ET3) both in the immature and mature compartments compared to glioma-bearing groups. Immunoblot data [Figure 2C and D] corroborated the flowcytometric finding and confirmed that the expression of Sca-1 increased significantly ( $P < 0.0001$ ) in dose-dependent manner in glioma associated BMHSCs of T11TS treated groups both in the LDC and HDC compared to that in HSCs of glioma-bearing ENU group both in LDC and HDC.

### Changes in the expression pattern of c-kit following T11TS therapy

C-Kit, a tyrosine kinase receptor for stem cell factor, acts as a unique marker for HSCs<sup>[52]</sup> and regulates the fate of HSCs<sup>[53]</sup>. Even small changes in the expression pattern of c-kit resulted in dramatic phenotypes and profoundly altered the developmental rhythm of hematopoiesis<sup>[54]</sup>.

Flowcytometric studies showed [Figure 3A and B] that following glioma induction, expression of c-kit in BMHSCs of ENU group, there was a remarkable ( $P < 0.001$ ) decrease in the expression level, both in LDC



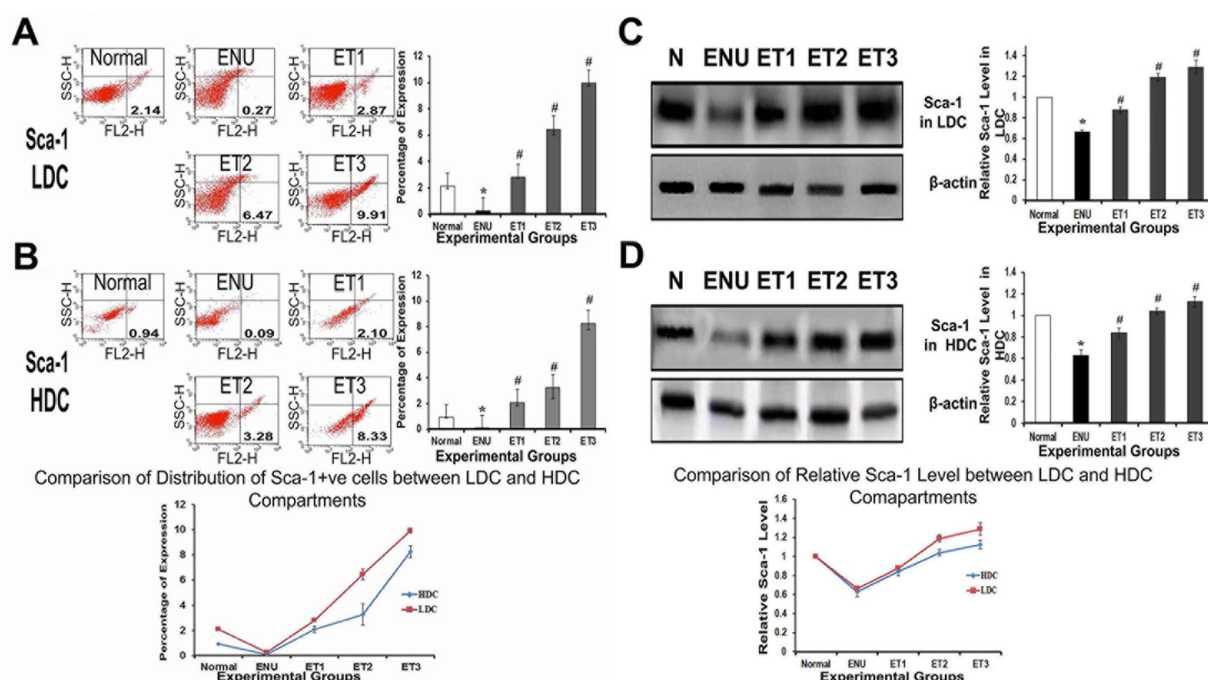


**Figure 1.** Comparative study of expression of CD34 in HSCs of five experimental animal groups, viz., normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of CD34 in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of CD34 percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of the expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group); B: Flowcytometric analysis of CD34 percent positive cells in the HDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group). Comparison of Distribution of CD34<sup>+</sup> Cells between LDC and HDC: The line diagram shows both the LDC and HDC HSCs there is a steep high rise in their expression level of CD34<sup>+</sup> cells following T11TS administration, but compared to HDC the LDC cells show the high level of proliferation as compared to ENU and normal groups probably hinting arousal of HSCs from niche as CD34 expressed mainly in primitive HSCs; C: representative images (100 $\times$  magnification) showing immunofluorescent staining of cell surface CD34 expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3) in both the LDC and HDC compartments. Each condition was observed in triplicate and six images were taken for each sample. Figures are representative of the group. Results were expressed as the percent CD34-positive cells out of the total number of cells counted for each experimental group. Column CD34: FITC-stained CD34 expression in bone marrow derived hematopoietic stem cells green in color due to the presence of CD34. Column DAPI: nuclei appear blue due to staining with DAPI. Column merged: FITC-stained fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean fluorescence intensity  $\pm$  SD of the respective group. At the bottom the comparison of the mean fluorescence intensity of CD34 positive cells between LDC and HDC has been also depicted. \*Significant ( $P < 0.001$ ) decrease in ENU compared with that of normal control group. #Significant ( $P < 0.001$ ) increase, when individually comparing T11TS treated groups with glioma-bearing ENU group in both the compartments. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

and HDC. T11TS administration in glioma-bearing animal significantly ( $P < 0.001$ ) up-regulated c-kit expression in glioma associated BMHSCs of both the groups in all three dose levels both in the mature and immature compartments as compared to the significantly low expression in the ENU induced glioma-bearing group.

Immunofluorescence imaging studies also confirmed our findings. Immunofluorescence staining and imaging [Figure 3C] of cytoplasmic c-kit expression of bone marrow-derived hematopoietic stem cells were evident from robust ( $P < 0.001$ ) decrease in cytoplasmic mean fluorescence intensity observed in bone marrow-derived hematopoietic stem cells of ENU group both in the LDC and HDC. Normal bone marrow-derived hematopoietic stem cells showed moderate fluorescence staining and hence medium



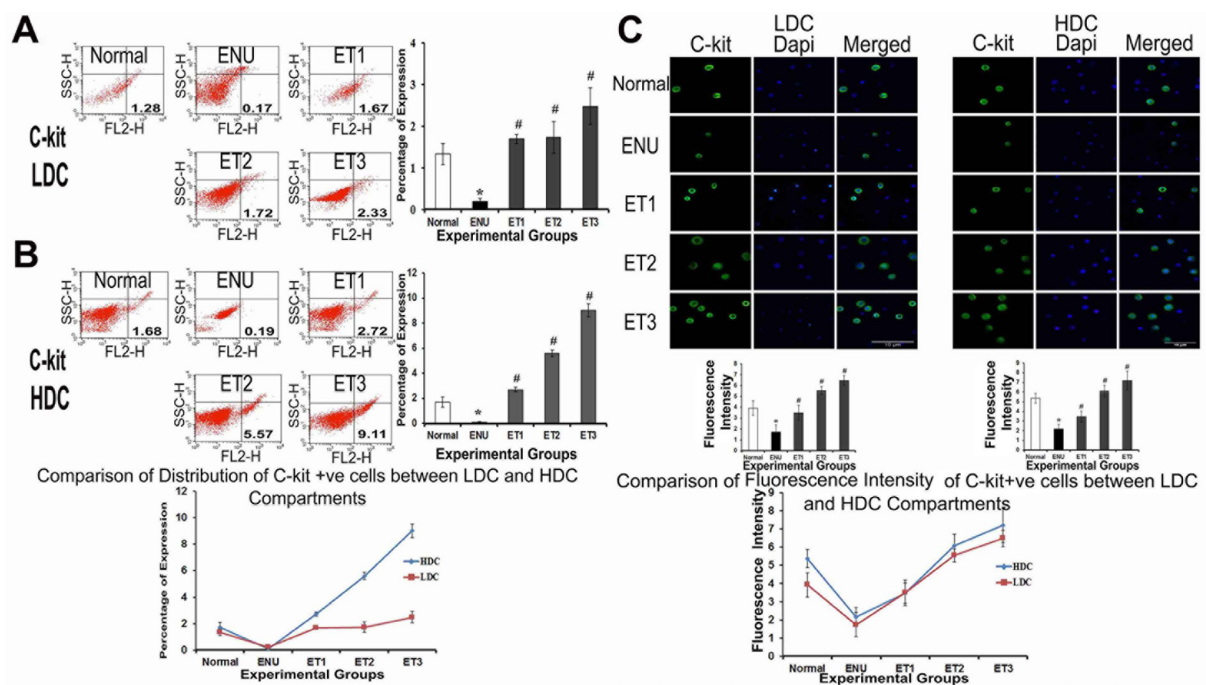


**Figure 2.** Comparative study of expression of Sca-1 in HSCs of five experimental animal groups, viz., normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of Sca-1 in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of Sca-1 percent positive cells in the low density compartment using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group); B: Flowcytometric analysis of Sca-1 percent positive cells in the high density compartment (HDC) using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group). Comparison of Distribution of Sca-1<sup>+</sup> cells between LDC and HDC: The line diagram shows both the LDC and HDC HSCs there are a steep high rise in their expression level of Sca-1 cells after T11TS administration, but compared to HDC the LDC cells show the high level of proliferation as compared to ENU and Normal groups probably hinting activated state of HSCs towards further maturity since Sca-1 expressed in both primitive and mature cells; C: Expression of Sca-1 in LDC was analyzed by immunoblotting using anti-Sca-1 specific antibody. The immunoblot shows band intensities for the Sca-1.  $\beta$ -actin was used as loading control and blots were reprobed with anti- $\beta$  actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams; D: expression of Sca-1 in HDC was analyzed by immunoblotting using anti-Sca-1 specific antibody. The immunoblot shows band intensities for the Sca-1.  $\beta$ -actin was used as loading control and blots were reprobed with anti- $\beta$  actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams. \*Significant ( $P < 0.001$ ) decrease in ENU compared with that of normal control group. #Significant ( $P < 0.001$ ) increase, when individually comparing T11TS treated groups with glioma-bearing ENU group. At the bottom, the comparison of expression of relative pixel intensities of Sca-1<sup>+</sup> cells between LDC and HDC. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

mean intensity both in the LDC and HDC. T11TS treatment caused a significant ( $P < 0.001$ ) increase in cytoplasmic c-kit expression in glioma-associated BMHSCs in all ET1, ET2 and ET3 groups, in dose dependent manner both in the LDC and HDC compared to that in hematopoietic stem cells of glioma-bearing ENU group in both the compartments.

### Modulation of Tie-2/Ang-1 signaling of bone marrow-derived hematopoietic stem cells

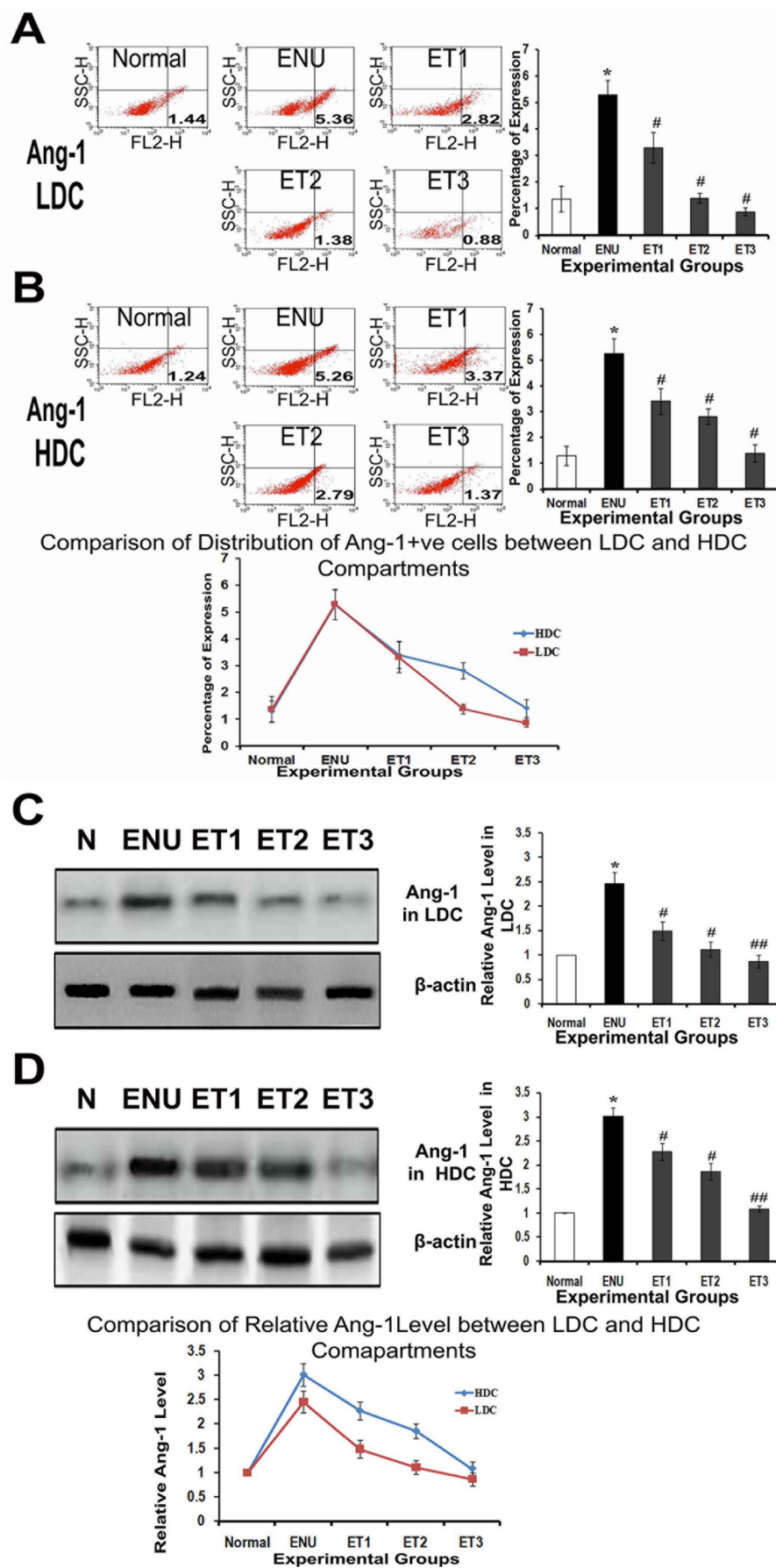
Regulatory role of Ang-1/Tie-2 signaling pathway during maturation of HSCs under various pathological stresses and correlation of activation of this pathway with quiescence state of HSCs within the bone marrow niche is gradually emerging. The status of maintenance of HSC at quiescence not only determines the lifelong self-renewing ability of HSC but also enhances their survivability by optimizing cell division<sup>[28]</sup>. In addition, up regulated Ang-1/Tie-2 signaling is believed to impart in the long-term repopulating ability of HSCs<sup>[55]</sup>.

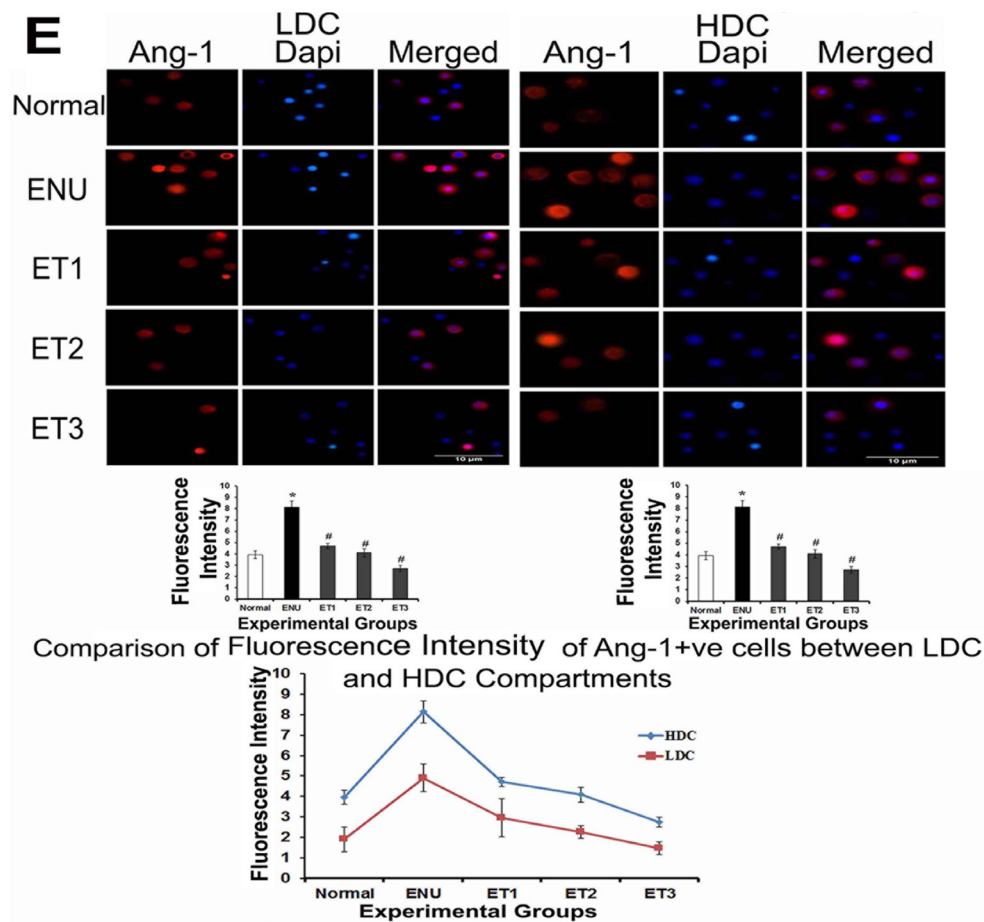


**Figure 3.** Comparative study of expression of c-kit in HSCs of five experimental animal groups, viz., the normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of c-kit in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of c-kit percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group); B: Flowcytometric analysis of c-kit percent positive cells in the high density compartments (HDC) using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group). Comparison of Distribution of c-kit<sup>+</sup> Cells between LDC and HDC. The line diagram shows both the LDC and HDC HSCs there is a steep high rise in their expression level of c-kit cells after T11TS administration, but compared to HDC the LDC cells shows the high level of proliferation as compared to ENU and normal since c-kit ligation opens up SCF signaling cascade which leads to lineage commitment of HSCs, so the results obtained here with also hints driving of both the group of cells towards lineage commitment; C: representative images (100 $\times$  magnification) showing immunofluorescent staining of cell surface c-kit expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3) in both the LDC and HDC compartment. Each condition was observed in triplicate and six images were taken for each sample. Figures are representative of the group. Results were expressed as the percent c-kit-positive cells out of the total number of cells counted for each experimental group. Column C-kit: FITC-stained c-kit expression in bone marrow derived hematopoietic stem cells green in color due to the presence of c-kit. Column Dapi: Nuclei appear blue due to staining with DAPI. Column Merge: Merged image FITC-stained fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity  $\pm$  SD of the respective group. At the bottom the comparison of the mean intensity of c-kit positive cells between LDC and HDC has been also depicted. \*Significant ( $P < 0.001$ ) decrease in ENU compared with that of normal control group. #Significant ( $P < 0.001$ ) increase, when individually comparing T11TS treated groups with glioma-bearing ENU group in both the compartments. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

Flowcytometric studies showed [Figure 4A and B] that following glioma induction Ang-1 expression BMHSCs of the ENU group in both the LDC and HDC compartments was remarkably ( $P < 0.001$ ) elevated compared to low basal expression in normal BMHSCs in both the LDC and HDC. T11TS treatment in glioma model, significantly ( $P < 0.001$ ) inhibited Ang-1 expression in glioma associated BMHSCs of LDC and HDC at all three (ET1, ET2 and ET3) dose levels in both the compartments as compared to the remarkably high expression in ENU group both in the LDC and HDC.

Relative band intensities of immunoblot experiment showed [Figure 4C and D] that expression of Ang-1 decreases significantly in BMHSCs both in the LDC and HDC in T11TS treated glioma associated BMHSCs in dose-dependent manner as compared to the significantly high level of expression in ENU induced glioma-





**Figure 4.** Comparative study of expression of Ang-1 in HSCs of five experimental animal groups, viz., the normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of Ang-1 in HSCs cells isolated from bone marrow of normal controls and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of Ang-1 percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group); B: Flowcytometric analysis of Ang-1 percent positive cells in the HDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form represented in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group). Comparison of distribution of Ang-1<sup>+</sup> cells between LDC and HDC: Certain groups of stem cells, which probably attach to the osteoblastic niche show higher expression of Ang-1<sup>+</sup> cells, indicating that quiescent state is being maintained in the Ang-1<sup>+</sup> cells to protect them from pathological insults and fine tuning the self-renewal capacity. Here high level of expression in the glioma group might be due to the attempt to save the stem cell pool from the devastating effect of a chemical carcinogen ENU. The subsequent treatment with three doses of T11TS brings back both the HDC and LDC cells to bear normal levels of Ang-1; C: expression of Ang-1 in LDC was analyzed by immunoblotting using anti-Ang-1 specific antibody. The immunoblot shows band intensities for the Ang-1.  $\beta$ -actin was used as loading control and blots were reprobated with anti- $\beta$  actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams; D: expression of Ang-1 in HDC was analyzed by immunoblotting using anti-Ang-1 specific antibody. The immunoblot shows band intensities for the Ang-1.  $\beta$ -actin was used as loading control and blots were reprobated with anti- $\beta$  actin antibody to establish equivalent loading. Bands were individually analyzed densitometrically and relative pixel intensities of individual, group were displayed in bar diagrams; E: representative images (100 $\times$  magnification) showing immunofluorescent staining of Ang-1 expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3 groups) in both the groups LDC and HDC. Each condition was observed in triplicate and six images were taken for each sample. Column Ang-1: TRITC stained Ang-1 expression in isolated BMHSC cells which appears red in color. Column Dapi: DAPI-stained nuclei appear blue. Column Merge: Merged picture TRITC fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity  $\pm$  SD of the respective group. \*Significant ( $P < 0.001$ ) increase in ENU compared with that of normal control group. #Significant ( $P < 0.001$ ) decrease, when individually comparing T11TS treated groups with glioma-bearing ENU group. At the bottom the comparison of the mean intensity of Ang-1 positive cells between LDC and HDC has been also depicted. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea



bearing group both in the LDC and HDC. Further, *in situ* immunofluorescence imaging studies [Figure 4E] of isolated BMHSCs showed a sharp ( $P < 0.001$ ) increase in mean fluorescence intensity of cytoplasmic Ang-1 in HSCs of ENU induced glioma-bearing group compared to that in normal BMHSCs in both the immature and mature compartments which showed moderate fluorescence staining and hence the moderate mean intensity of fluorescence. However, T11TS treatment was able to bring down the fluorescence intensity of cytoplasmic Ang-1 in dose dependent manner ( $P < 0.001$ ) in glioma-associated HSCs of ET1, ET2, and ET3 groups compared to that in HSCs of the glioma-bearing ENU group.

### T11TS therapy inhibits Tie-2 expression

Flowcytometric studies showed [Figure 5A and B] that there was simultaneous remarkable ( $P < 0.001$ ) elevated expression of Tie-2 in BMHSCs following glioma induction of the ENU group in both the compartments LDC and HDC compared to low basal expression in normal BMHSCs in both the LDC and HDC. T11TS therapy in glioma model, which significantly ( $P < 0.001$ ) decreased Tie-2 expression in glioma associated BMHSCs of both LDC and HDC compartment in all the three ET1, ET2 and ET3 dose levels in dose dependent manner as compared to the remarkably high expression in ENU group both in the LDC and HDC.

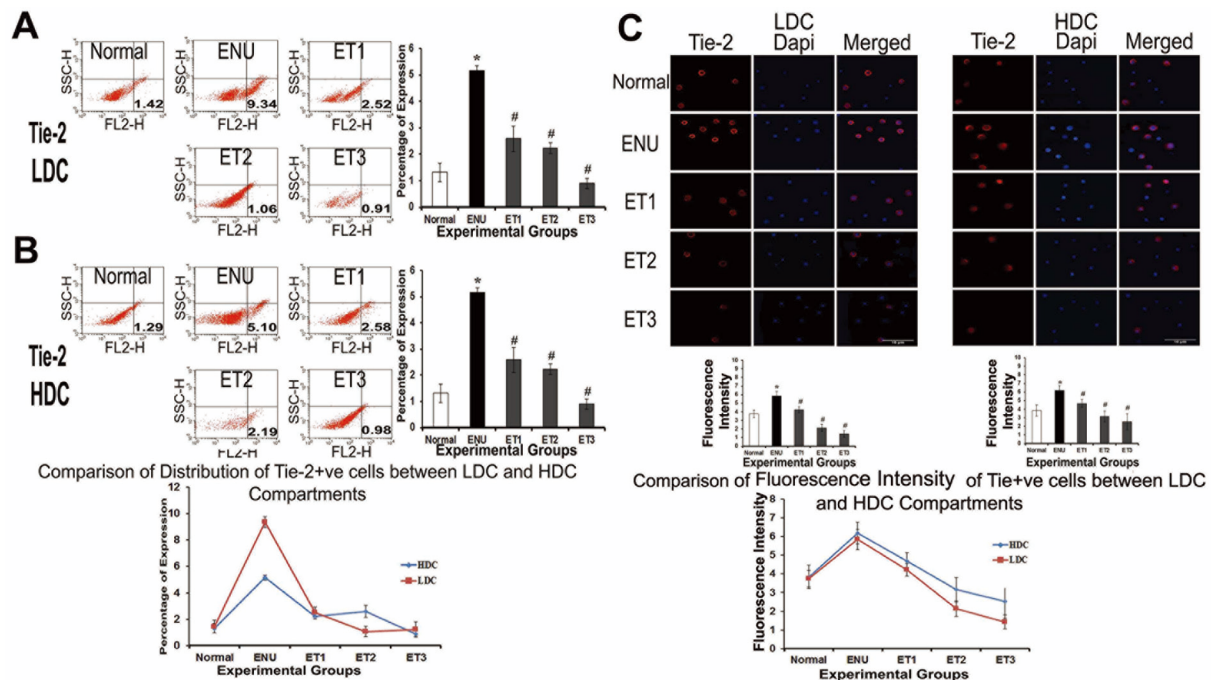
*In situ* immunofluorescence staining of isolated BMHSCs of rats from different experimental groups showed that glioma induction in the ENU group caused a remarkable increase in Tie-2 expression [Figure 5C] in HSCs within bone marrow both in the LDC and HDC when compared to that in isolated HSCs from a normal control group in both the compartments. T11TS therapy caused a significant ( $P < 0.001$ ) dose-dependent down regulation of Tie-2 expression in glioma-bearing HSCs within the bone marrow of T11TS treated groups both in the LDC and HDC compartments.

Tie-2 expression pattern in glioma-associated BMHSCs as observed from flowcytometric studies concurred with the in-situ immunofluorescence imaging results.

## DISCUSSION

There is global immune suppression during glioma growth. Intraperitoneal administration of ENU in 5-6 days old neonatal rats produce grade IV glioma after 5-6 months of age. The salient features of ENU induced full-blown grade IV glioma, have been elaborately elucidated histologically<sup>[56]</sup>, by Glial fibrillary acidic protein (GFAP) staining<sup>[57]</sup>, by cell proliferation index studies<sup>[58]</sup>, and by *in situ* immunofluorescences<sup>[59]</sup>. The published literature has also conclusively documented the gliomagenic global immune suppression on glioma-bearing animals and the beneficial immunomodulatory effect of T11TS immunotherapy<sup>[60-63]</sup>. Following T11TS administration, there is profound immune stimulation which in turn results in the proliferation of the immunocytes and rejuvenates immune suppressive state of the malignant glioma<sup>[61,62,64-67]</sup>. Therefore, the novelty of our work is that this is the first reporting of the status of HSCs during glioma occurrence and thereafter following immunotherapy, which has not been explored so far. Both, in HDC comprised of mature cells and LDC composed of relatively immature cells, the suppressive effect on HSCs during a chemical carcinogen-induced glioma condition has been documented and reversal of this suppressive situation after T11TS administration has also been noted.

The Sialomucin CD34 is a critical marker for primitive HSCs, the expression pattern of which appears to be responsible for most of the early phases of hematopoietic activity<sup>[50,68]</sup>. Our study demonstrated that T11TS therapy significantly stimulates the level of expressions of CD34 both at the LDC and HDC in BMHSCs of glioma-bearing rats. The diminished level of CD34<sup>+</sup> on HSCs of glioma-bearing animals indicates a diminution of primitive HSCs and suppression of normal hematopoiesis during glioma progression, which might be associated with the accelerated pre-mature apoptosis of the hematopoietic machinery due to the invading glioma<sup>[41]</sup>. From our experiments, it was delineated that LDC in all the experimental groups contains



**Figure 5.** Comparative study of expression of Tie-2 in HSCs of five experimental animal groups, viz., normal, healthy control rats (N), glioma-bearing rats (ENU), and rats having received the first (ET1), second (ET2) and third (ET3) doses of T11TS. Flowcytometric studies of the expression of Tie-2 in HSCs cells isolated from bone marrow of normal control and of glioma-bearing rats before and after T11TS treatment have been shown. A: Flowcytometric analysis of Tie-2 percent positive cells in the LDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group); B: Flowcytometric analysis of Tie-2 percent positive cells in the HDC using BD Cell Quest Pro Software by using scatter from a single representative experiment. The percentage of expression refers to the percent positive cells out of 10,000 cells analyzed represented in graphical form in bar diagram. Column values represent mean  $\pm$  SD ( $n = 6$  animals per group). Comparison of Distribution of Tie-2<sup>+</sup> Cells between LDC and HDC: Tie-2 which is ligand to Ang-1 and is expressed on HSCs is highly expressed in the LDC group compared to the HDC in ENU treated groups, though both groups show up-regulated expression compared to the normal group. This shows that the immature HSCs are more protected than the mature cells at HDC during the ENU insult. T11TS down regulates the higher expression of both the groups below normal levels, indicating that T11TS revive the quiescent state of HSCs driving them towards differentiation and self-renewal properties; C: representative images (100 $\times$  magnification) showing immunofluorescent staining of Tie-2 expression on bone marrow derived hematopoietic stem cells of normal (N) and glioma-bearing rats before (ENU) and after T11TS treatment (ET1, ET2 and ET3 groups) in both the groups LDC and HDC. Each condition was observed in triplicate and six images were taken for each sample. Column Tie-2: TRITC stained Tie-2 expression in isolated BMHSC cells which appears red in color. Column Dapi: DAPI-stained nuclei appear blue. Column Merge: Merged picture TRITC fluorescence intensity of each group was analyzed with Nikon's Nis - Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity  $\pm$  SD of the respective group. \*Significant ( $P < 0.001$ ) increase in ENU compared with that of normal control group. #Significant ( $P < 0.001$ ) decrease, when individually comparing T11TS treated groups with glioma-bearing ENU group. At the bottom the comparison of the mean intensity of Tie-2 positive cells between LDC and HDC has been also depicted. HSCs: hematopoietic stem cells; LDC: low density compartment; HDC: high density compartments; ENU: N-ethyl-N-nitrosourea

more immature CD34<sup>+</sup> stem cells than the HDC [Figure 1A and B] which is perhaps due to migration and spontaneous differentiation of CD34<sup>+</sup> stem cells from the HDC compartment towards further maturity. Moreover, compartmental mobilization of bone marrow cells under various pathological/physiological conditions is a routine phenomenon<sup>[69]</sup>. The result suggests that T11TS is able to stimulate glioma-associated HSC's revival from the niche with the regeneration of the CD34<sup>+</sup> cells<sup>[41]</sup>.

With the aim to delineate mechanistic insights of T11TS immunotherapy on hematopoietic signaling system during gliomagenesis, we have further investigated another two important markers of HSCs, Sca-1 and c-kit known for their long-term stem cell activity<sup>[70]</sup>.



Sca-1 or lymphocyte activation protein-6A (Ly-6 A/E)<sup>[71]</sup> not only marks various developmental stages of HSCs<sup>[25]</sup> but also plays an indispensable regulatory role during self-renewal for normal hematopoiesis<sup>[25,72]</sup>. In our experiment [Figure 2A and B], it has been observed that compared to the normal group, glioma induction causes a significant decrease of the level of Sca-1 both at LDC and HDC. Evaluation with normal counterparts, HDC population was shown to be lower than the population at LDC which might be due to impairment of normal transition of immature HSCs from LDC to HDC during glioma growth, ultimately resulting in a decrease in the maturity of Sca-1<sup>+</sup> HSCs due to a negative feedback effect. During T11TS administration, there was a gradual increase in expressions of Sca-1 at all dose levels, both at LDC and HDC and a sharp increase in Sca-1<sup>+</sup> cells after third dose of T11TS with higher level of expression in the LDC group as pointed out in the line diagram [Figure 2]. T11TS therapy not only promotes self-renewal of both the population of Sca-1<sup>+</sup> cells but also hints towards further maturity. Phenotypic expression of Sca-1 distinguishes the stem cell compartment from committed myeloid and lymphoid progenitors, as its rapid down regulation was observed during differentiation<sup>[73]</sup>.

The proto-oncogene c-kit, a cell surface receptor tyrosine kinase and its ligand stem cell factor (SCF) play an essential role in intra-marrow hematopoiesis during adulthood<sup>[74]</sup>. C-kit is exclusively expressed in primitive HSCs to impart essential regulatory function during the early stages of hematopoiesis<sup>[75-77]</sup>. *In vivo* blocking of interaction between c-kit and SCF demote HSCs self-renewal by hindering the release of HSCs from the bone marrow. In our experiment [Figure 3A and B], ENU mediated glioma induction causes a significant decrease of the expression level of c-kit both in the LDC and HDC as compared to the normal group, but after treatment with T11TS, the level gradually increased with the consecutive doses (ET1 and ET2), and the level of receptor expression is significantly higher than the glioma group and even higher than the normal group at the third dose (ET3) both in the LDC and HDC. In glioma-bearing animals, the lower expression level in the HDC as compared to LDC might be due to the preferential killing of comparatively mature cells that are expressing constitutively active c-kit in terms of the active form of the cell cycle in the HDC sparing immature more primitive dormant progenitors<sup>[78]</sup>. Further, the comparative line diagram [Figure 3] shows simultaneous up-regulation of c-kit<sup>+</sup> cells in the HDC as compared to LDC of T11TS treated groups, indicating that the primitive immature progenitors have a lower c-kit expression as compared to their mature descendants in the HDC having higher c-kit expression. The observation further confirms the notion that as immature primitive cells enter the phase of maturation, divide actively and initiate the phase of differentiation, the expression of c-kit intensifies<sup>[79,80]</sup>. This is perhaps due to the fact that the high expression pattern of c-kit opens up SCF signaling cascade to guide the HSCs towards lineage commitment where SCF may influence the migration of hematopoietic stem cells to their ultimate destinations of development.

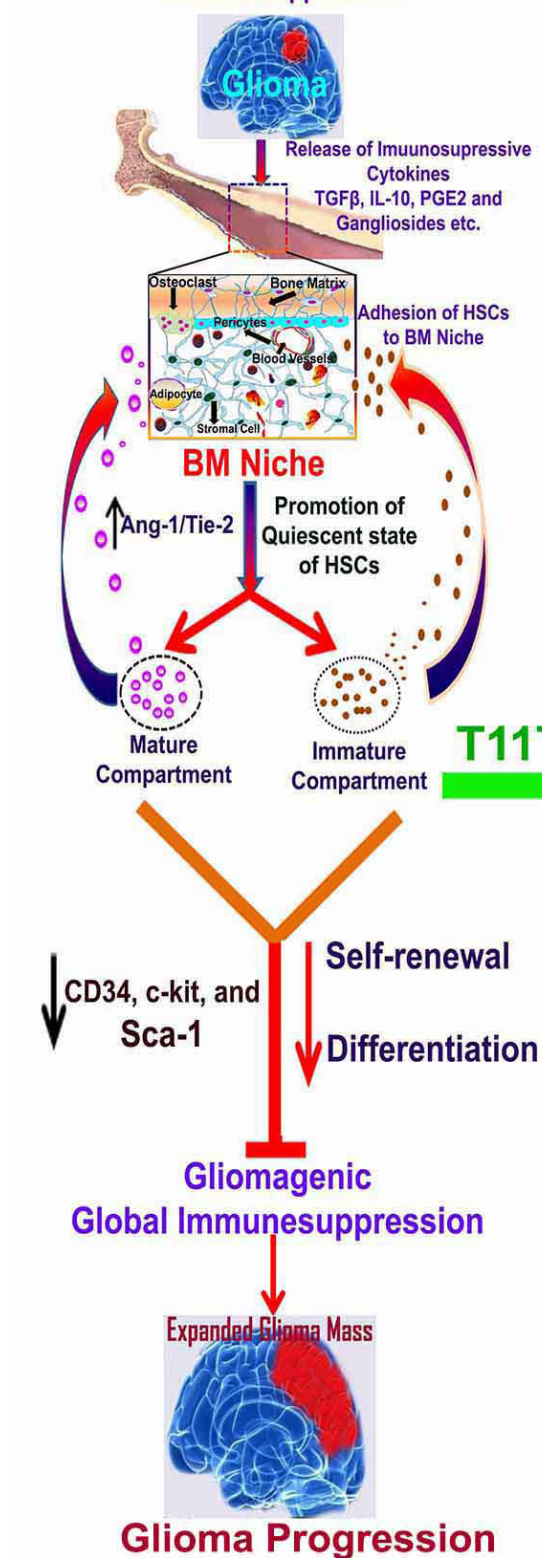
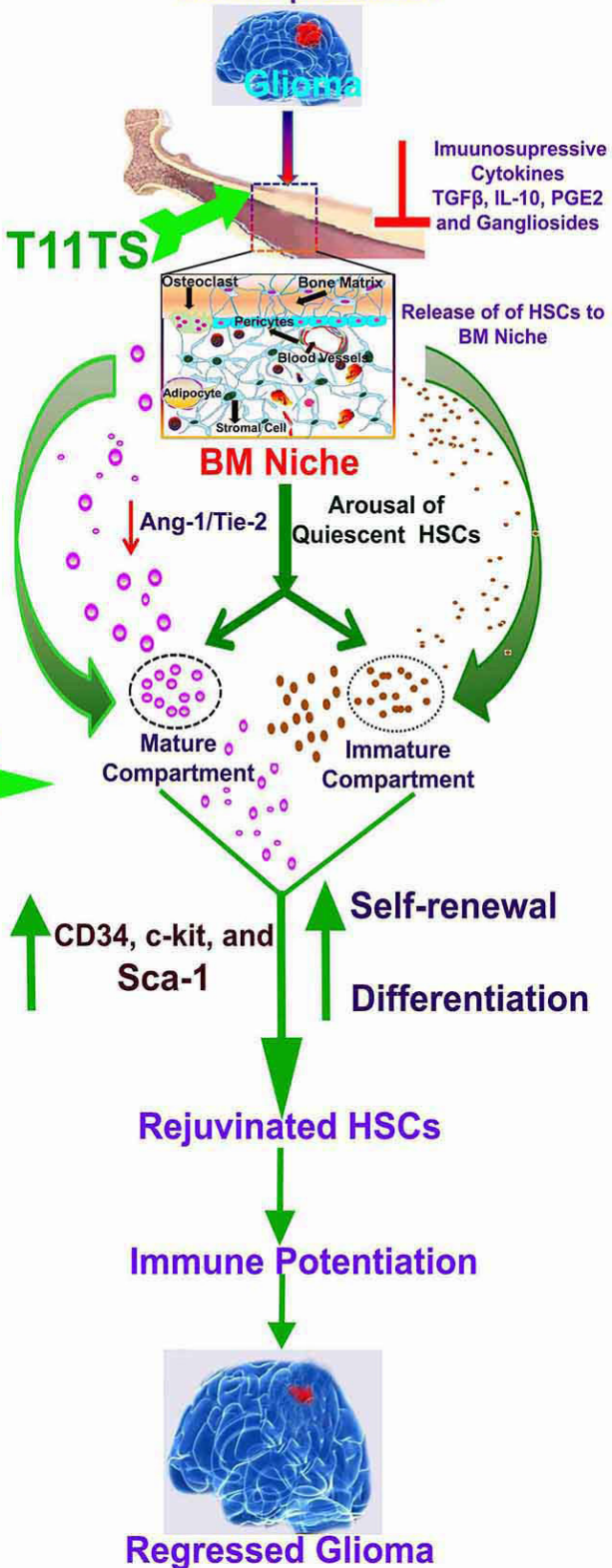
Both short-term and long-term repopulating HSCs reside within the CD34<sup>+</sup>, Sca-1<sup>+</sup> and c-kit<sup>+</sup> bone marrow population<sup>[81]</sup>. During normal physiological condition, a fine tune between self-renewal and differentiation is able to maintain the constant number of HSCs. However, disease conditions such as anemia, cancer or during myelotoxic chemotherapy have been evidence of alteration of total stem cell number indicating that the counterbalance between self-renewal and differentiation can reconcile as per physiological needs<sup>[82,83]</sup>. This pliability is most likely achieved by molecular crosstalk between stem cells and their specific microenvironment called the niche. This reciprocal intracellular interaction between HSCs and bone marrow niche also regulates the status of HSCs and influences their surface phenotypes. On the other hand, the quiescent state of hematopoietic stem cells is thought to be critical in sustaining a self-renewing HSC compartment for life and to shield the stem cell pool from premature exhaustion during various pathological conditions. Indeed, the maintenance of quiescent state and slow cell-cycle progression of HSCs could directly be linked to the robust reconstitution ability and long-term sustainability<sup>[84]</sup>. In addition, quiescent HSC populations are resistant to 5-fluorouracil-induced

myelosuppression<sup>[28]</sup>, suggesting that the quiescence of HSCs is closely associated with the protection of the HSC pool from the various stresses induced by myelotoxic insults. However, precise regulatory mechanisms of niche cell-HSCs remain elusive.

Ang-1 expressed by niche cell is one of the indispensable interacting molecules which function as an autocrine activating factor for Tie-2 signaling in HSCs and their interaction activates  $\beta$ 1-integrin and N-cadherin, enhances quiescent state and facilitates long-term repopulating activity of HSCs by facilitating induced adhesion to bone and homing during the physiological crisis. The critical regulatory role of Ang-1/Tie-2 signaling in the maintenance of HSCs quiescence and promoting their adhesion to bone marrow niche, resulting in protection of HSCs compartment from myelosuppressive insults has been documented before<sup>[28]</sup>. Furthermore, the mechanistic insight of this protective interaction against apoptosis by activating the PI3K pathway has also been addressed<sup>[85]</sup>. We have also documented the inhibitory role of T11TS on glioma mediated pro-angiogenic Ang-1/Tie-2 signaling within brain endothelial cells ultimately restraining pro-tumorigenic angiogenesis in rodent glioma model<sup>[86]</sup>. Although the mechanism through which glioma modulated activation of Ang-1/Tie-2 signaling within bone marrow niche and their correlation with gliomagenic global immune suppression remain to be elucidated, we hypothesize that gliomagenic up regulation of Ang-1/Tie-2 interaction disrupts the normal hematopoietic synchrony and ultimately affect the lymphohematopoiesis.

Over expression of Ang-1<sup>+</sup> cells at osteoblastic niche signifying the quiescent state of HSCs as a protective barrier during pathological conditions to fine tune the self-renewal capacity. Here [Figure 4A and B], high level of expression in the glioma group might be due to the attempt to save the stem cell pool from the devastating effect of the chemical carcinogen ENU. The subsequent treatment with three doses of T11TS brings back both the HDC and LDC cells to near normal levels of Ang-1. The above mentioned effect of Ang-1 on HSCs in the glioma-bearing state is definitely mediated by over expression of Tie-2 as Ang-1 is believed to be the dominant ligand for Tie-2 receptor in LT-BMHSCs and contributed in sustenance of stem cell activities within the bone marrow niche<sup>[87]</sup>. In our finding [Figure 5A and B] higher expression of Tie-2 at LDC compared to HDC in ENU treated groups as compared to normal group hint to their protective effect on the immature HSCs than the mature cells at HDC during ENU insult. T11TS down regulates the higher expression of Tie-2 from both the mature and immature hematopoietic cells below normal levels, indicating that T11TS revive the quiescent state of HSCs driving them towards normal hematopoiesis of reconstitution and self-renewal.

In summary, our current investigation clearly demonstrated for the first time, that during glioma condition, there was significant disruption in early phases of hematopoietic cell signaling. Our phenotypic studies of the two compartments of HSCs comprising immature LDC and mature HDC cells indicate that during glioma development all the receptors in both the compartments are drastically reduced due to reversion to the quiescent state of the particular population and also cell loss due to apoptosis as hinted in our recent publication<sup>[41]</sup>. Their regenerative capacities were all renewed by T11TS treatment rendering “Therapeutically equipped HSC”. Primarily the immature cells had been stimulated from the quiescent state as shown by CD34 up-regulation. Next, they were driven towards maturity as denoted by Sca-1 up-regulation and lastly c-kit up regulations indicating the signaling involvements towards progenitor cell development. Up-regulation of the niche receptor Ang-1 and its counter-receptors Tie-2 of HSCs in the glioma group and drastic down regulation of both the stem cell pool by T11TS confirms the protective effect of T11TS to facilitate revival from the quiescent state of HSCs driving them towards differentiation and self-renewal properties [Figure 6]. Our findings should provide helpful guidance for the therapeutic utilization of T11TS during glioma and will also facilitate the understanding of how HSCs behave during gliomagenic immunological shock.

**Glioma mediated Hibernation of HSC and Global Immune Suppression****T11TS mediated rejuvenation of HSC leading to immune potentiation**

**Figure 6.** Proposed pathway of gliomagenic alteration of expression pattern of important early phase phenotypic markers in ENU induced glioma-bearing rats and their modulation following T11TS therapy hints towards immunocompetence against immune suppressive glioma. ENU: N-ethyl-N-nitrosourea

## DECLARATIONS

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### Authors' contributions

Conducted experiments and provided results: Mondal S, Datta A, Hazra I, Omar Faruk SM, Moitra S, Chaudhuri S, Nath L, Das PK, Basu AK

Co-wrote the first draft: Mondal S

Edited the manuscript: Chaudhuri S

Co-supervised aspects of the project: Tripathi SK

Provided overall supervision of the project, edited and finalized the manuscript: Chaudhuri S

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

All studies involving animals were reviewed and approved by Institutional Ethical Committee at Calcutta School of Tropical Medicine, Kolkata, West Bengal, India, monitored by Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India (CPCSEA) regulations.

### Consent for publication

Not applicable.

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## Erratum

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# Erratum: Characteristics and predictive biomarkers of drug resistant epilepsy -- study in Georgia

Neuroimmunology and Neuroinflammation Editorial Office

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The Neuroimmunology and Neuroinflammation Editorial Office would like to report an abbreviation-related error in the published article<sup>[1]</sup>.

The first sentence of paragraph 2 in Introduction part “Approximately 2/3 of patients with epilepsy become seizure-free following correct diagnosis and on appropriate treatment with antiepileptic drugs automated external defibrillator (AED)” has been replaced by “Approximately 2/3 of patients with epilepsy become seizure-free following correct diagnosis and on appropriate treatment with antiepileptic drugs (AED)”.

During the production, there was a mistake in matching up the abbreviation “AED” with corresponding words and it has been corrected. We apologize for any inconvenience caused to the readers by this change. The change does not affect the scientific results.

The [original article](#) was published on 27 Sep 2017.

## REFERENCE

1. Alkhidze M, Lomidze G, Kasradze S, Tsiskaridze A. Characteristics and predictive biomarkers of drug resistant epilepsy -- study in Georgia. Neuroimmunol Neuroinflammation 2017;4:191-8.



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Review

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# Glioma associated microglia/macrophages, a potential pharmacological target to promote antitumor inflammatory immune response in the treatment of glioblastoma

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## ABSTRACT

Glioma associated microglia/macrophages (GAMs) constitute the largest proportion of glioma infiltrating cells, particularly in high grade tumors (i.e., glioblastoma). Once inside the tumors, GAMs usually acquire a specific phenotype of activation that favors tumor growth, angiogenesis and promotes the invasion of normal brain parenchyma. Therefore, treatments that limit or prevent GAMs' recruitment at the tumor site or modulate their immune activation promoting antitumor activities are expected to exert beneficial effects in glioblastoma. In the present paper, we aim at the revision of pharmacological strategies that interfere with GAMs' function and are currently proposed as an alternative/additional option to current approved cytotoxic regimens.

**Keywords:** Glioblastoma, macrophages, microglia, metalloproteases, pro-inflammatory activation, pro-tumor functions, glioma associated microglia/macrophages targeted therapies, pharmacotherapy

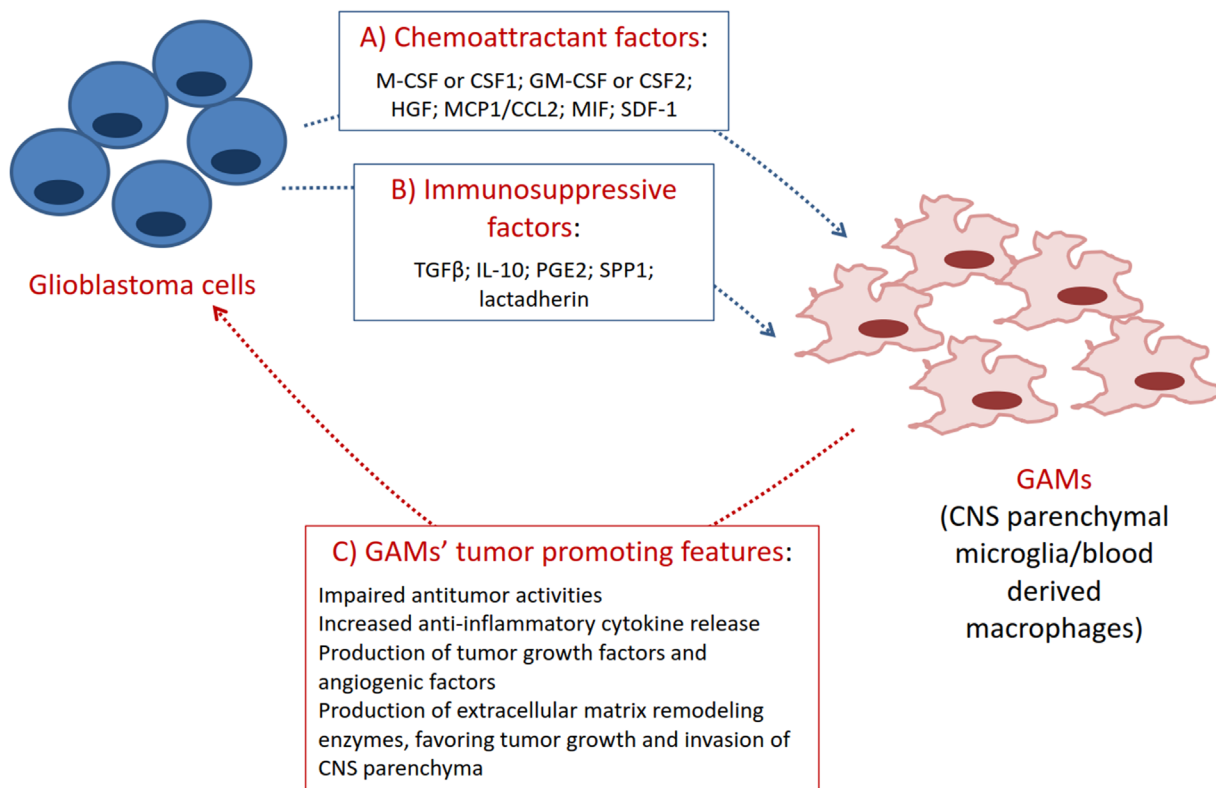
## INTRODUCTION

Glioma associated microglia/macrophages (GAMs) constitute the largest proportion of tumor infiltrating cells. They are less abundant in low grade gliomas, but constitute up to 30% of the entire tumor mass in



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**Figure 1.** Cross-talk between glioblastoma cells and GAMS. A: Glioblastoma cells produce several chemoattractant factors which promote the recruitment of microglia and macrophages at the tumor site, GAMS; B: once inside the tumor, GAMS are exposed to immunosuppressive/antiinflammatory factors and are reprogrammed towards phenotypes that sustain tumor growth, progression and invasion; C: the most relevant tumor promoting features of GAMS are presented. M-CSF: macrophage colony stimulating factor; CSF1: macrophage colony stimulating factor; GM-CSF: granulocyte/macrophage colony stimulating factor; CSF2: granulocyte/macrophage colony stimulating factor; HGF: hepatocyte growth factor; MCP-1/CCL2: monocyte chemoattractant protein 1/chemokine (C-C motif) ligand 2; MIF: macrophage inhibitory factor; SDF-1: stromal-derived factor-1; TGF β : transforming growth factor β; IL: interleukin; PGE2: prostaglandin E2; SPP1: osteopontin; GAMS: glioma associated microglia/macrophages; CNS: central nervous system

glioblastoma (IV grade glioma)<sup>[1]</sup>. On the basis of largescale genomic analyses, glioblastoma can be classified into at least four distinct molecular subtypes<sup>[2]</sup>, among which the mesenchymal subtype tends to have the most relevant immune component<sup>[3]</sup>. Microglial cells, scattered in normal brain parenchyma, are recruited at the tumor site by glioblastoma-secreted chemoattractant factors<sup>[4,5]</sup> [Figure 1], while peripheral blood-derived macrophages, normally found in the perivascular space, meninx and choroid plexus, accumulate in glioblastoma through breakdown of the blood-brain-barrier (BBB)<sup>[1]</sup> particularly in high grade glioma<sup>[6]</sup>. Iba1<sup>+</sup> cells were consistently detected in a group of 41 glioblastoma specimens, showing preferentially an amoeboid phenotype toward the tumor center and a ramified morphology in the periphery of the tumors<sup>[7]</sup>. In addition, markers suggesting both pro- and anti-tumoral properties of GAMS were detected. A significant proportion of cells expressing the cluster of differentiation (CD) 163 and the inducible nitric oxide synthase (iNOS) was found in the tumor parenchyma together with a wider distribution of arginase 1 positive cells<sup>[7]</sup>. GAMS are frequently detected in the perivascular niche of tumor blood vessels, and their number increases with tumor progression<sup>[8]</sup>. As shown in Figure 1, invading microglia/macrophages play a critical role in the regulation of glioma biology, including tumor growth, progression and invasion<sup>[8]</sup>. Consistently, depletion of microglia/macrophages *in vivo* experimental models significantly reduced tumor growth<sup>[8-12]</sup>, holding the potential to ameliorate the outcome of current available therapies.

In this regard, standard treatment for glioblastoma includes maximal surgical resection (whenever feasible), followed by radiotherapy and concurrent treatment with temozolomide plus additional 6 cycles of adjuvant temozolomide<sup>[13]</sup>. Despite such multimodal approach, the average survival of patients diagnosed with glioblastoma remains low (14-16 months), with better outcomes observed when tumors display O<sup>6</sup>-methylguanine

DNA-methyltransferase promoter methylation<sup>[13]</sup>. In fact, most glioblastoma tumors tend to recur after being surgically removed. This is partly due to the highly infiltrative nature of these cancer cells, so that radical surgery is difficult to achieve. On the other hand, tumors can regenerate from glioblastoma cancer stem cells (GSCs) that are usually resistant to radio- and chemotherapy. Treatment guidelines for recurrent disease are less defined and may include a second surgery, re-irradiation, or re-exposure to temozolomide at standard dose. Other options comprise systemic chemotherapy with one nitrosourea drug, i.e., carmustine, lomustine, or fotemustine, and in the United States, the monoclonal antibody against vascular endothelial growth factor-A (VEGF-A) bevacizumab<sup>[13]</sup>. Finally, several targeted therapies have been tested in clinical trials with limited beneficial effects. Interestingly, we observed using primary cultures of rat microglial cells that temozolomide did not reduce microglial cell viability after 24 h treatments in the  $\mu\text{mol/L}$  (clinically relevant) dose range, albeit it significantly increased intracellular protein content<sup>[14]</sup>. Notably, resistance to anti-angiogenic therapy, i.e., bevacizumab, appears to be mediated by changes in the glioblastoma's microenvironment, including the extent of myeloid cell infiltration as well as their biological activities<sup>[15-17]</sup>. In preclinical models of glioblastoma, it has been shown that ionizing radiations increase the recruitment of myeloid cells with a pro-tumorigenic phenotype at the tumor site, contributing to disease recurrence<sup>[18]</sup>. Taken together, the evidence suggest a possible involvement of GAMs in the response to standard treatments. Therefore, glioma associated myeloid cells can be envisioned as an alternative or an ancillary pharmacological target to improve the clinical outcome of current available therapies. Noteworthy, the glioblastoma microenvironment includes also other immune cells, namely regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), that concur to the establishment of an immunosuppressive environment, impairing the effector function of infiltrating T cells and natural killer cells and facilitating tumor growth<sup>[19]</sup>. Therefore, a comprehensive understanding of these different components of the patients' immune system endowed in the tumor microenvironment is necessary to develop therapeutic strategies that increase anti-tumor immunity and clinical benefits. In the present paper, we aim at the revision of pharmacological strategies that interfere with GAMs' function, i.e., cell recruitment at the tumor site, cell inflammatory activation and immune function, and the extracellular matrix remodeling promoted by GAM-secreted factors. For recent advances on the biology of MDSCs and Tregs in the glioblastoma microenvironment and their potential role as therapeutic targets, we refer the readers to other review articles<sup>[20,21]</sup>.

## DRUGS TARGETING GAMs' FUNCTION FOR THE TREATMENT OF GLIOBLASTOMA

### Drugs that interfere with GAMs' recruitment at the tumor site

Microglial cells are recruited at the tumor site by several chemoattractant factors which are produced and released by tumoral cells<sup>[4,5]</sup>. One of the first identified GAMs' chemoattractant factor is the hepatocyte growth factor<sup>[22]</sup>, which binds to and activates the tyrosine kinase receptor, c-Met. The latter plays a role both on microglial motility and cell proliferation<sup>[22]</sup>. Other glioma-released chemoattractant factors are the myeloid colony stimulating factors (CSFs), i.e., the macrophage colony stimulating factor (M-CSF or CSF1)<sup>[23]</sup>, the granulocyte/macrophage colony stimulating factor (GM-CSF or CSF2)<sup>[24]</sup>. These factors signal through activation of two different receptors<sup>[25]</sup>. The M-CSF receptor (CSF1R) is a homodimeric type III receptor, encoded by the *FMS* proto-oncogene, with intrinsic tyrosine kinase activity, whereas the GM-CSF receptor (CSF2R) is a heterodimer composed of a specific ligand-binding subunit (the  $\alpha$ -chain) and a common  $\beta$ -chain. The latter is the signal transduction subunit and is shared with the receptors for interleukin (IL)-3 and IL-5. Activation of the CSF2R is known to stimulate at least three pathways: the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase pathway<sup>[25]</sup>. In addition, the monocyte chemotactic proteins (MCPs), particularly MCP-1/chemokine (C-C motif) ligand 2 (CCL2)<sup>[26,27]</sup>, and the stromal-derived factor-1 (SDF-1)<sup>[28]</sup>, have shown to play a role in the recruitment of microglial cells to the tumor site [Figure 1]. In addition a substantial number of peripherally derived macrophages can be consistently detected in glioma GL261 implanted tumors, since the early phases of disease<sup>[29]</sup>. Once inside the tumors, GAMs usually acquire a specific phenotype of activation<sup>[30]</sup> that favors tumor growth, angiogenesis and promotes the invasion of normal brain pa-



renchyma<sup>[5]</sup>. Therefore, pharmacological treatments that prevent or reduce GAMs' recruitment at the tumor site are expected to exert beneficial effects in glioblastoma. In this regard, it has been shown that the immunosuppressant agent cyclosporine A (CsA), a drug normally used in clinical practice, significantly reduces the number of infiltrating microglia/macrophages in implanted glioma tumors. This effect, together with a modulation of GAM's inflammatory activation, results in a significant reduction of tumor growth<sup>[9]</sup>. However, chronic immunosuppression associated with systemic use of CsA increases the risk of developing tumors, and may probably limit the use of this drug for the treatment of glioblastoma. In addition, several tumor favoring mechanisms are associated with CsA, including increased production of transforming growth factor  $\beta$  (TGF $\beta$ ) and VEGF together with an inhibitory action on the DNA repairing ability of the cells<sup>[31]</sup>. In the human U-87 glioma cell line, CsA significantly reduced the expression level of the human microRNA (miRNA, miR-195, together with the modulation of several other miRNAs<sup>[32]</sup>. Interestingly, miR-195 seems to play a tumor suppressor function in both glioma cell lines and human gliomas<sup>[33,34]</sup>. On the other hand, the nuclear factor of activated T cells (NFAT1), i.e., the main intracellular target of CsA, appears to be a crucial regulator of glioma invasion-related genes. Thus, a direct inhibition of NFAT1 activity in glioma cells can limit their ability to infiltrate normal brain parenchyma, and may be considered as a potential adjuvant therapy for glioblastoma<sup>[35]</sup>.

Interestingly, novel compounds, interfering with known chemoattractant factors, are in different stages of development for the treatment of glioblastoma. For example, it has been shown that the CSF-1R inhibitor BLZ945, which blocks the signaling pathways activated by M-CSF, significantly increased survival in different preclinical models of glioblastoma<sup>[36]</sup>. This pharmacological treatment induced the regression of established tumors in engineered mice and abated tumor growth in human xenografts. The drug is a small molecular weight CSF-1R inhibitor, with optimal BBB penetration properties. However, despite the chemoattractant properties of M-CSF and its established role in promoting macrophage survival, BLZ945 did not reduce the number of tumor infiltrating microglia/macrophages in these models. GAMs appeared indeed protected from BLZ-induced cell death by glioma-secreted cytokines such as GM-CSF, interferon  $\gamma$  (IFN $\gamma$ ) and the C-X-C motif chemokine 10 (CXCL10)<sup>[36]</sup>. On the other hand, BLZ945 modulated the inflammatory activation of GAMs, favoring their antitumor activities which explains the beneficial effects observed with the treatment (see next section). In a different preclinical model of glioblastoma, consisting of tumors derived by implantation of GSCs lacking tumor suppressor phosphatase and tensin homolog, p53 and neurofibromin 1 (NF1), BLZ945 efficiently blocked GAMs' recruitment at tumor site together with reducing tumor growth<sup>[37]</sup>. Interestingly, a first-in-human study employing BLZ945 (NCT02829723) is currently ongoing. It is a phase I/II with BLZ945 given as a single agent or in combination with PDR001 [a novel monoclonal antibody against the immune checkpoint programmed death-1 (PD-1) receptor, by Novartis Oncology], which aims at the characterization of the safety, tolerability, pharmacokinetics, pharmacodynamics, and anti-tumor activity of BLZ945 in adult patients with advanced solid tumors. Moreover, another selective CSF-1R inhibitor (PLX3397) has been recently tested in a phase II clinical trial in patients affected by recurrent glioblastoma (NCT01349036). The drug was well tolerated, showed good BBB penetration, and reduced the amount of Iba1<sup>+</sup> cells within the tumors. However, no significant improvement in the progression free survival compared with historical controls was observed in PLX3397 treated patients<sup>[38]</sup>. Moreover, it has been recently shown that genetic reduction of MCP-1/CCL2 significantly reduces macrophage infiltration within the tumors extending the survival time of tumor bearing animals<sup>[39]</sup>. However, previous attempts to block MCP-1/CCL2 with monoclonal antibodies demonstrated modest clinical efficacy. The drugs were instead effective in combination with temozolomide, significantly increasing mice survival<sup>[27]</sup>. Interestingly, it has been shown that the production of MCP-1 by glioma cells can be efficiently reduced by non-cytotoxic drugs, including the antibiotic minocycline, the angiotensin II receptor inhibitor telmisartan and the bisphosphonate zoledronic acid<sup>[40]</sup>. These drugs have a good BBB penetration and will be tested in combination as an ancillary therapy to improve the outcome of currently approved cytotoxic regimens.

Recently, a small molecular weight inhibitor of the AXL receptor tyrosine kinase has been shown to exert



relevant antiproliferative effects on different preclinical models of glioblastoma. The drug, namely BGB324 (also known as R428) significantly increased neurological free survival particularly in the group of mice bearing high-AXL expressing tumors<sup>[37]</sup>. In addition, BGB324 treatment reduced the amount of infiltrating CD45<sup>+</sup> leukocytes and CD11b<sup>+</sup> GAMs. Interestingly, the anti PD-1 inhibitor nivolumab increased the protective effects of BGB324, and effectively prolonged the survival of tumor bearing mice<sup>[37]</sup>. Nivolumab *per se* displayed no survival benefits in these animals, while increasing both AXL kinase activity and GAMs' tumor infiltration. In line with these observations, a phase III clinical trial (NCT02017717) set to compare the efficacy and safety of nivolumab administered alone versus bevacizumab in patients diagnosed with recurrent glioblastoma failed to demonstrate its efficacy<sup>[19]</sup>. Immune PD-1 check point inhibitors, including nivolumab, have proven efficacy in various malignancies and the number of clinical approved indications is constantly increasing<sup>[41]</sup>. The use of these drugs is associated with specific toxicities, often termed immune-related adverse events. The most common side effects involve the skin, colon, endocrine organs and liver. Rarely, neurological complications have been described<sup>[41]</sup>, including recent case reports on nivolumab-induced autoimmune encephalitis<sup>[42]</sup> and progressive multifocal leukoencephalopathy<sup>[43]</sup>.

Finally, microglial/macrophages' infiltration of GSC-derived tumors was efficiently blocked by the integrin inhibitor arginine-glycine-aspartic acid (RGD) peptides albeit interfering with GSC-secreted periostin<sup>[44]</sup>. Consistently, genetic ablation of periostin reduced GAMs' recruitment at tumor site and modulated their immune functions, thus inhibiting tumor growth and increasing survival of glioma bearing animals. Similar beneficial effects were expected by pharmacological inhibition of integrin signaling pathways in human glioblastoma. However, despite promising phase I/II results, a recent phase III clinical trial failed to demonstrate clinical efficacy of cilengitide, a cyclic RGD pentapeptide that selectively inhibits the  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins when added to standard temozolomide treatment in glioblastoma patients<sup>[45]</sup>. A possible explanation for these negative findings can be retrieved in part in the unfavorable pharmacokinetic profile of cilengitide<sup>[46]</sup>. In fact, the relevance of the signaling pathways downstream the integrin receptors,  $\alpha v\beta 3$  and  $\alpha v\beta 5$ , is further supported by a recent proteomic analysis of the glioma secretome. These data suggest the involvement of osteopontin (SPP1) and lactadherin in the reprogramming of GAMs' immune responses towards pro-tumoral functions via integrin signaling<sup>[47]</sup>.

### Drugs that interfere with GAMs' inflammatory activation and immune function

Under the influence of glioma cells, the antitumor functions of GAMs appear mostly suppressed. As shown in Figure 1, tumor cells indeed produce several immunosuppressive molecules, such as TGF $\beta$ , IL-10, and various prostaglandins (i.e., prostaglandin E2, PGE2), thus favoring the acquisition of a pro-tumorigenic phenotype of activation by GAMs<sup>[30,48,49]</sup>. Pharmacological strategies that promote antitumor activities of GAMs, i.e., production of cytotoxic molecules and increased phagocytosis, or that reduce the release of pro-tumorigenic (i.e., growth factors) may exert beneficial effects in glioblastoma. In this regard, amphotericin B (AmpB), an antifungal compound clinically used to treat life-threatening fungal infections<sup>[50]</sup>, has been shown to promote macrophage activation via toll like receptor activation and increase pro-inflammatory cytokine release<sup>[51]</sup>. In view of these properties, AmpB was recently tested in preclinical models of gliomas. In an experimental model consisting of human-derived GSC tumors implanted in nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice, systemic administration of AmpB significantly reduced tumor growth and increased animal survival<sup>[52]</sup>. The drug did not exert direct anti-tumor activity on GSCs *in vitro* and its pharmacological benefits *in vivo* were abated by depletion of myeloid cells. This suggests that the beneficial effects of AmpB were mediated by modulation of GAMs' functions. Increased tumor infiltration of Iba1<sup>+</sup> microglial cells and macrophages was detected in AmpB treated animals. This effects has been recently confirmed using ultrasmall iron oxide nanoparticles as contrast agents for magnetic resonance imaging, in order to detect monocyte infiltration into brain tumors<sup>[53]</sup>. In addition, tumor infiltrating Iba1<sup>+</sup> cells in response to AmpB showed a significant up-regulation of iNOS, that most likely results in increased production of cytotoxic nitric oxide (NO)<sup>[52]</sup>. Beneficial effects of AmpB were also observed in immunocompe-

tent C57BL/6 mice, against highly aggressive tumors derived from enriched stem-like CD133<sup>+</sup> GL261 glioma cells. Notably, the antitumor effects of AmpB *in vivo* are achieved with lower doses than those maximally tolerated in humans.

Another promising class of therapeutics for the treatment of glioblastoma are the inhibitors of the mechanistic target of rapamycin (mTOR) kinase and/or other related kinases. The mTOR kinase is a central regulator of several intracellular processes related to cellular growth, metabolism, and proliferation<sup>[54]</sup>. Robust evidence have highlighted the crucial role of this pathway in glioblastoma biology, together with the demonstration of significant antiproliferative effects obtained by its pharmacological inhibition<sup>[55]</sup>. Several drugs targeting this activity are currently in clinical development for the treatment of different types of cancer<sup>[56]</sup>, including those with an optimal pharmacokinetic profile for the treatment of glioblastoma<sup>[56,57]</sup>. Notably, we have shown that inhibition of mTOR activity in rat microglial cells can promote their antitumor properties while restricting pro-tumorigenic features<sup>[58]</sup>. Therefore, mTOR inhibitors have the potential to target both glioblastoma and GAMs' functions. Similarly, the chemokine receptor C-C chemokine receptor type 5 (CCR5) inhibitor maraviroc, in the same *in vitro* model, showed both direct antiproliferative activities on rat glioma C6 cells together with immune modulatory actions on glioma stimulated rat microglial cultures<sup>[59]</sup>.

Interestingly, both glioma and infiltrating GAMs express the Ca<sup>2+</sup>-activated K<sup>+</sup> channels (KCa3.1), whose inhibition using 1-(2-chlorophenyl) diphenylmethyl-1H-pyrazole (TRAM-34) induced a switch of GAMs toward a pro-inflammatory, antitumor phenotype<sup>[60]</sup>. In addition, *in vivo* treatments with TRAM-34 significantly decreased the extent of tumor growth in glioma-bearing mice<sup>[60]</sup>. Moreover, stimulation of microglia with pro-inflammatory IL-12 is associated with increased phagocytic activity<sup>[61]</sup>. Consistently, intracranial injection of a recombinant adeno-associated viral vector (rAAV2) encoding for IL-12 augmented the brain levels of IL-12 and IFN $\gamma$  in tumor-bearing animals, favoring microglial infiltration into the tumor and restoring their antitumor functions. Increased immune activation of GAMs significantly reduced tumor growth and prolonged animal survival time<sup>[62]</sup>. Similarly, systemic administration of miR-142-6p, whose expression level is consistently downregulated in GAMs, extended animal survival in different glioma models. These beneficial effects relied on reduced GAMs' infiltration at the tumor site and increased antitumor activities<sup>[63]</sup>. Inhibition of the C-X-C chemokine receptor type 4 (CXCR4) by a newly synthesized receptor antagonist, peptide R, reduced tumor growth, glioma cell invasiveness, and intratumor vessel formation while directing GAMs' immune activation toward a pro-inflammatory/antitumor phenotype<sup>[64]</sup>. Notably, SDF-1 suppression in a murine glioma resulted in delayed tumor growth and invasiveness, lower microvascular density, and higher density of microglia/macrophages in non-hypoxic compared to hypoxic regions. These findings suggest that tumor-secreted SDF-1 stimulates glioma invasiveness and recruitment of GAMs towards hypoxic areas<sup>[65]</sup>. In addition, it has been recently shown that the antitumor activity of vosaroxin, a first in class cytotoxic agent that intercalates DNA and inhibits topoisomerase II, are also linked to increased recruitment of myeloid cells at the tumor site together with an augmented pro-inflammatory activation<sup>[66]</sup>. Likewise, the antitumor effects of chlorogenic acid (5-caffeoylquinic acid) (CHA) found in pre-clinical models of glioblastoma were associated with increased antitumor immune activations of GAMs. CHA is phenolic compound found in the human diet, in coffee, apples, pears and in green tea<sup>[67]</sup>. Finally, a recent paper describes the beneficial effects of a single chain antibody (X7Ab) directed against the chemokine receptor ACKR3/CXCR7. Reduction of tumor growth and improved survival were observed *in vivo* in different pre-clinical models of glioblastoma, particularly when X7Ab was used in combination with standard doses of temozolomide. Interestingly, increased mean fluorescence intensity of classical activated (major histocompatibility complex class II, MHCII<sup>+</sup>) tumor infiltrating macrophages was detected, suggesting augmented pro-inflammatory (i.e., antitumor) activation of these cells within the tumor microenvironment<sup>[68]</sup>.

### Drugs that interfere with matrix remodeling promoted by GAM-secreted factors

Besides their immune functions which may either restrict or favor astrocyte malignant transformation,

GAMs are directly involved in the degradation of the extracellular matrix. Thus, these cells are key regulators of a central process involved in the expansion of tumors as well as in the invasion of normal brain parenchyma<sup>[69]</sup>. In fact, microglial cells significantly increase the invasive phenotype of GL261 glioma cells *in vivo*<sup>[70]</sup>. Consistently, the invasiveness of glioma cells is diminished in microglial-depleted organotypic brain slices inoculated with GL261 glioma cells<sup>[71]</sup>. Matrix metalloproteases (MMPs), i.e., the enzymes involved in the remodeling of the extracellular environment<sup>[72]</sup>, are largely produced by tumor cells, infiltrating microglia/macrophages, or other infiltrating leukocytes, particularly at the invasive tumor edge facilitating tumor growth and invasion<sup>[71,73,74]</sup>. As detailed in our recent review<sup>[4]</sup>, a complex crosstalk exists between glioma cells and infiltrating GAMs which increases the activity of MMP enzymes, including MMP-2 and MMP-9. Notably, the latter is over-expressed in GAM cells sorted from human glioblastoma tissues<sup>[75]</sup>.

Consistently, several pharmacological treatments displayed beneficial effects in glioblastoma by limiting the release of MMPs. For example, minocycline, a highly lipophilic tetracycline antibiotic with a good BBB penetration property, reduced the expression of MT1-MMP in invading microglia/macrophages by suppressing p38 MAPK activation<sup>[76]</sup>. The drug also reduced secretion of MMP-9<sup>[75]</sup> and other pro-inflammatory cytokines from microglia and tumor cells resulting in an overall decrease of glioblastoma cell migration<sup>[76]</sup>. Notably, minocycline is also able to reduce MCP-1 secretion by glioblastoma cells, thus potentially limiting GAMs' recruitment at tumor site (as discussed above). The same inhibitory effects on MT1-MMP were displayed *in vitro* by the lipid lowering agent, atorvastatin<sup>[77]</sup>. In addition, propentofylline, an atypical methylxanthine with central nervous system (CNS) glial modulating and anti-inflammatory actions, significantly reduced tumor growth by targeting microglial production of MMP-9. The drug restricted also the migratory capacity of both glioma CNS-1 cells and microglia *in vitro*<sup>[78]</sup>. Invasion and infiltration of the normal brain parenchyma interfere with radical surgical resections of glioblastoma, that often recur after the first aggressive treatment. Pharmacological reduction of glioma cell motility and invasiveness thus hold the potential to improve the outcome of current therapeutic approaches, by limiting the infiltration extent of normal brain parenchyma<sup>[69]</sup>.

### Other features of GAMs

*In vitro*, microglia co-cultured in the presence of glioma cells appear to be morphologically activated although phagocytosis is largely impaired<sup>[10]</sup>. Nevertheless, another promising therapeutic approach for the treatment of glioblastoma consists in the use of nanoparticles which are internalized by GAMs increasing their antitumor immune activation<sup>[79,80]</sup>. Moreover, GAMs produce a vast array of growth and angiogenic factors which further sustain proliferation of tumor cells<sup>[8,48,52]</sup> as well as tumor vessel formation<sup>[81]</sup>. Interestingly, genetic and pharmacological ablation in GAMs of neuropilin 1, a co-receptor that amplifies signaling through the VEGF-A and TGFβ pathways, is associated with reduced glioma growth and blood vessel formation and increased survival time of glioma bearing mice<sup>[82]</sup>.

## CONCLUSION

GAMs represent the most relevant population of tumor infiltrating cells that significantly contribute to the pathogenesis of glioblastoma by favoring tumor growth and invasion of the normal brain parenchyma. Pre-clinical evidence supports the notion that GAMs are a viable pharmacological target whose function can be modulated in order to prevent their pathological activation. Current available data, summarized in Table 1, suggest that the immune activation of GAMs can be genetically or pharmacologically modulated so that these cells can be efficiently instructed to perform anti-tumor activities. In addition, it is possible to control their recruitment at the tumor site, and the production of extracellular matrix remodeling enzymes, thus limiting tumor growth and the ability to infiltrate normal brain parenchyma. One of the main limitations to systemic chemotherapy for glioblastoma is represented by the inability of most drugs to effectively penetrate the BBB and achieve cytotoxic concentrations in the cerebrospinal fluid and brain parenchyma. In fact, sev-

**Table 1. Drugs targeting GAMs' functions within the glioblastoma microenvironment**

Drug name and approval status	Drug properties	Molecular target	Pharmacological actions on GAMs	Other effects	Clinical outcome	Ref.
<b>Preclinical evidence</b>						
<b>Amphotericin B</b> Approved for clinical use by FDA and in EU member states	Small MW compound	Toll-like receptors	↑ GAMs' tumor infiltration ↑ GAMs' antitumor immune activation ↑ iNOS expression and NO production	No direct antiproliferative effects on GSCs <i>in vitro</i>	↓ Tumor growth ↑ Survival	[52, 53]
<b>Cyclosporine A</b> Approved for clinical use by FDA and in EU member states	Small MW compound	Calcineurin/NFAT1	↓ GAMs' tumor infiltration ↓ IL10, ARG1 and GM-CSF ↓ MMP2	↑ TGFβ and VEGF ↓ DNA repair ↓ miR195 and other miRNAs	↓ Tumor growth Potential tumor promoting activities	[9,31,32]
<b>Minocycline</b> Approved for clinical use by FDA and in EU member states	Small MW compound	p38-MAPK	↓ MT1-MMP, ↓ MMP-9 production by GAMs ↓ Tumor cells' migration	↓ Pro-inflammatory cytokines by microglia ↓ MCP-1 by glioma cells	↓ Tumor growth ↑ Survival	[40,75,76]
<b>Nivolumab</b> Approved for clinical use by FDA and EMA	Biologic (mAb)	PD-1	↑ GAMs' tumor infiltration ↑ AXL kinase activity	↑ Protective effects of BG324	No survival benefits <i>per se</i>	[37]
<b>mTOR kinase inhibitors</b> Approved/investigational drugs	Small MW compound	mTOR kinase	↑ Pro-inflammatory activation of microglia <i>in vitro</i>	Direct antiproliferative effects	↓ Tumor growth	[55,57,58]
<b>BGB324 (R428)</b> Investigational	Small MW compound	Receptor tyrosine kinase AXL	↓ CD11b+ GAMs' tumor infiltration ↓ CD45+ leukocyte tumor infiltration		↑ Survival	[37]
<b>BLZ945</b> Investigational	Small MW compound	CSF-1R	↑ survival of GAMs ↑ GAMs' phagocytic activity ↓ GAMs' protumor immune activation	↑/↓ GAMs' tumor infiltration	↓ Tumor growth ↑ Survival	[36,37]
<b>CHA</b> Investigational	Small MW compound	STAT factors	↑ GAMs' antitumor immune activation		↓ Tumor growth	[67]
<b>Propentofylline</b> Investigational	Small MW compound	Phosphodiesterase	↓ MMP-9 by GAMs	↓ Migratory capacity of microglia and glioma	↓ Tumor growth	[78]
<b>TRAM-34</b> Investigational	Small MW compound	KCa3.1 channels	↑ GAMs' antitumor immune activation		↓ Tumor growth	[60]
<b>Vosaroxin</b> Investigational	Small MW compound	DNA and TOPO-II	↑ GAMs' tumor infiltration ↑ GAMs' antitumor immune activation		↓ Tumor growth	[66]
<b>Peptide R</b> Investigational	Synthetic peptide	CXCR4	↑ GAMs' antitumor immune activation	↓ Glioma invasiveness, ↓ Intratumor vessel formation	↓ Tumor growth	[64]
<b>RGD peptides</b> Investigational	Synthetic peptides	Integrins	↓ GAMs' tumor infiltration ↑ GAMs' antitumor immune activation	↓ GSC-secreted periostin	↓ Tumor growth	[44]
<b>IL-12 or rAAV2-mediated IL-12</b> Investigational	Biologic (protein or engineered viral vector)	IL-12 receptor	↑ GAMs' tumor infiltration ↑ GAMs' antitumor immune activation ↑ GAMs' phagocytic activity	↑ IFNγ and IL-12 intratumoral levels induced by rAAV2.	↓ Tumor growth ↑ Survival	[61,62]
<b>miR-142-6p</b> Investigational	Biologic (Synthetic oligonucleotide)	mRNA	↑ GAMs' tumor infiltration ↑ GAMs' antitumor immune activation		↓ Tumor growth ↑ Survival	[63]
<b>X7Ab</b> Investigational	Biologic (single-chain antibody)	ACKR3 / CXCR7	↑ GAMs' antitumor immune activation	Increased therapeutic effects of TMZ	↓ Tumor growth ↑ Survival	[68]

**Clinical evidence**

<b>Nivolumab</b> FDA and EMA approved	Biologic (mAb)	PD-1		Phase III	No superior survival [19] vs. bevacizumab
<b>BLZ945/PDR001</b> Investigational	Small MW compound/ Biologic (mAb)	CSF-1R/PD1		Phase I/II	Clinical trials. gov
<b>PLX3397</b> Investigational	Small MW compound	CSF-1R	↓ Iba1+ cells within the tumors	Phase II (recurrent glioblastoma)	No significant effects on PFS compared with historical controls [38]
<b>Cilengitide</b> Investigational (in combination with TMZ)	Synthetic cyclic RGD pentapeptide	avβ3 and avβ5 integrins		Phase III	No superior survival [45,46] vs. TMZ alone

In the table we reported the main features of drugs that interferes with biological functions of GAMs (name, characteristics and molecular target) together with the pharmacological actions on GAMs and the clinical outcomes on glioblastoma. Drugs are listed based on the level of evidence, i.e., preclinical (*in vitro* and *in vivo*) or clinical testing, according to the following criteria: (1) approved for clinical use (1st, small molecular weight compounds, 2nd biologics); and (2) investigational drugs (1st, small molecular weight compounds, 2nd biologics). ↑: increased; ↓: reduced. AmpB: amphotericin B; ARG1: arginase 1; BBB: blood-brain-barrier; CD: cluster of differentiation; CCL2: chemokine (C-C motif) ligand 2; CCR5: C-C chemokine receptor type 5; CHA: chlorogenic acid (5-caffeoylquinic acid); CsA: cyclosporine A; CSFs: colony stimulating factors; CSF1: macrophage colony stimulating factor; CSF1R: M-CSF receptor; CSF2R: GM-CSF receptor; CSF2: granulocyte/macrophage colony stimulating factor; GAMs: glioma associated microglia/macrophages; GM-CSF: granulocyte/macrophage colony stimulating factor; GSCs: glioblastoma cancer stem cells; JAK: Janus kinase; HGF: hepatocyte growth factor; Iba1: ionized calcium-binding adapter molecule 1, i.e., a specific myeloid lineage marker; IFN  $\gamma$ : interferon  $\gamma$ ; IL: interleukin; iNOS: inducible nitric oxide synthase; mAb: monoclonal antibody; MAPK: mitogen-activated protein kinase; MCP: monocyte chemotactic protein; M-CSF: macrophage colony stimulating factor; MDSCs: myeloid-derived suppressor cells; mTOR: mechanistic target of rapamycin kinase; MMP: matrix metalloprotease; miRNA, or miR: microRNA; NFAT1: nuclear factor of activated T cells; MW: molecular weight; PFS: progression free survival; PG: prostaglandin; PI3K: phosphoinositide 3-kinase; PD-1: programmed death-1; rAAV2: recombinant adeno-associated viral vector; SDF-1: stromal-derived factor-1; SPP1: osteopontin; STAT: signal transducer and activator of transcription; TGF  $\beta$ : transforming growth factor  $\beta$ ; TMZ: temozolomide; TOPO-II: topoisomerase-II; TRAM-34: 1-(2-chlorophenyl) diphenylmethyl-1H-pyrazole; Tregs: regulatory T cells; VEGF-A: vascular endothelial growth factor-A; X7Ab: single chain antibody

eral strategies attempt to overcome this restriction such as improved drug formulation (i.e., nanoparticles or lipid based formulation), local drug delivery (including gene therapy<sup>[61,62]</sup>), or transient BBB permeabilization<sup>[83,84]</sup>, to name a few. Among the above mentioned drugs, minocycline and rapamycin for example, have increased BBB penetration properties; and novel mTOR inhibitors with improved pharmacokinetic properties are also under development. It is possible to envision the use of pharmacological compounds, targeting GAMs' functions, as a complement to current available therapeutic approaches.

**DECLARATIONS****Authors' contributions**

Conceived the paper and wrote the primary draft: Dello Russo C  
Contributed to the literature revision and manuscript editing: Cappoli N  
Read and approved the final manuscript: Dello Russo C, Cappoli N

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**Conflicts of interest**

All authors declared that there are no conflicts of interest.



## Ethical approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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Meeting Abstracts

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## 2018 Mental Health and Neurology Conference

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### 1. Evaluation of hypermethylation and expression pattern of glutamate and dopamine receptors genes in patients with schizophrenia

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**Aim:** Schizophrenia (SCZ) is a type of psychotic disorders that affects 1% population. Dopamine and glutamate are the major neurotransmitters in brain and their receptors are associated with the number of psychotic disorders such as schizophrenia. The aims of the present study were to analyze methylation and expression profile of dopamine and glutamate receptors genes in patients with SCZ.

**Methods:** Methylation-specific polymerase chain reaction (MS-PCR) was used to estimate promoter hypermethylation of dopamine and glutamate receptors genes on 81 isolated genomic DNA, from peripheral blood of individuals with schizophrenia and 71 healthy control subjects. In addition, real-time reverse transcription - PCR was used to estimate mRNA levels in 34 blood samples of healthy controls and cases.

**Results:** Methylation of GRM2 and GRM5, highly increased the risk of schizophrenia in comparison to reference unmethylated pattern [OR = 2.82, (1.05-7.75),  $P = 0.038$ ], [OR = 12.09, (1.84-79.57),  $P = 0.0001$ ] respectively. Regarding the dopamine receptors genes, promoter methylation of DRD4 and DRD5 genes were statistically different ( $P < 0.05$ ) in cases when compared with healthy controls in blood samples. Outcomes of expression analysis revealed statically significant difference between cases ( $n = 17$ ) and health controls ( $n = 17$ ) regarding relative genes expression of GRM2, GRM5 and GRIA3, DRD2, DRD4 and DRD5 ( $P < 0.0001$ ).

**Conclusion:** To the best of our knowledge, this is the first report which indicates the methylation status and expression profile of GRs and DRs genes with the risk of SCZ. These outcomes suggested more attention to the effect of epigenetic variations in development of SCZ in further investigations.



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## 2. Epilepsy: the bridge between psychiatry and neurology

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Epilepsy is a group of neurological disorders characterized by epileptic seizures<sup>[1]</sup>. In 2000 BC, epilepsy was considered as a demonic possession. Hippocrates (460-370 BC) proposed that epilepsy was a medically treatable problem originating in the brain<sup>[2]</sup>. Epilepsy as a mental disorder was proposed by Morel (1857). He stated that epilepsy is caused by hereditary degeneration, which resulted in progressive intellectual and moral degeneration. The concept of epilepsy as a neurologic disorder was introduced by Robert Bentley Todd (1844) who developed the neuronal discharges theory<sup>[3]</sup>. Epilepsy reflects brain dysfunction, that can affect the mind and behavior. While the epileptic seizures themselves are episodic, the mental and behavioral changes continue, in many cases, interictally<sup>[4]</sup>. Many symptoms of neurologic or psychiatric illness - such as cognitive impairment, depression, anxiety, attention deficits, and migraine - occur more frequently in people with epilepsy than in the general population<sup>[5]</sup>. The discipline of neurology emerged from “nervous disorders” or neuropsychiatry in the late 19th century, when vascular theories of epilepsy predominated. By the turn of the 19th century psychiatry and neurology were diverging and epilepsy remained to some extent in both disciplines<sup>[6]</sup>. It was only in the middle of the 20th century with the development of electromagnetic theories of epilepsy that the concept of epilepsy per se as a neurological disorder was finally adopted in international classifications of disease. In 1960, WHO determined that epilepsy as a neurological (not mental) disease, and epilepsy with additional mental syndromes, was the province of psychiatry<sup>[6]</sup>. At the beginning of the 21st century and the centenary of the ILAE, psychiatry and neurology have been converging again, led in some respects by epilepsy, which has provided several useful models of mental illness and a bridge between psychiatry and neurology<sup>[6]</sup>.

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## 3. Low level laser therapy: improved regeneration of injured sciatic nerve by He-Ne laser

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Neuronal tissue is one of the most important tissues of the body which recovers extremely sluggishly after being injured. Inability of the neural tissue to regenerate in response to trauma and the disability which

ensues has prompted a lot of efforts in search for new means of neural rehabilitation. Low-energy He-Ne laser irradiation has been proposed as a sensible choice<sup>[1]</sup>. In the current experiment, sciatic nerves of 20 adult rats were crushed surgically. The subjects were randomly assigned into case and control groups. The cases received daily laser irradiation ( $\lambda = 65 \text{ nm}$ ) for 4 min<sup>[2]</sup>. The muscular function of the rats was tested by angle board every 3 days, from the 3rd day postoperation. On the 27th day, all the rats were sacrificed and the manipulated sciatic nerves were excised and studied histologically<sup>[3]</sup>. The results showed significant improvement of neural structure and muscular function in laser treated rats over the controls, as revealed by angle board testing and microscopic examination<sup>[4]</sup>. We conclude that low-energy He-Ne laser greatly restores crushed sciatic nerves in rats and can be considered for clinical trials.

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## 4. Building health research capacity in Africa for UHC: the profile of stroke in Jos, North-Central Nigeria

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**Aim:** To determine the risk factors associated with stroke, assess the case fatality 90 days post stroke, determine the bad prognostic factors of stroke and assess the sensitivity and specificity of clinical sub-typing of stroke using the WHO and Siriraj stroke scoring tools.

**Methods:** A longitudinal cohort study with a 90-day follow up for secondary outcome was carried out on Stroke patients admitted into the neurology unit of Jos University teaching Hospital over a 2-year period, September 1st 2016 to August 2018.

**Results:** A total of 246 stroke patients were admitted during the study period. Males were 131 (53.3%) and females 115 (46.6%) with an age range of  $59.5 \pm 13.1$  for males and  $56.7 \pm 14.2$  for females. Hypertension (81.7%), obesity (80.9%), dyslipidemia (54.5%), alcohol consumption (24.8%), carotid plaques (19.5%), cardiac disease (19.1%) and diabetes mellitus (18.5%) were the commonest risk factors for stroke. The 90 days fatality for stroke was 22%; however, 37% became disabled and unable to carry out activities of daily living without support. Significant predictors of mortality and morbidity were: coma, elevated glycated hemoglobin, cardiac disease, HIV infection and high National institute of health stroke score. WHO clinical stroke sub-typing showed a sensitivity of 54.3% and a specificity of 86.3% while Siriraj has a sensitivity of 87.9% and specificity of 84.9% for ischemic stroke, however, for hemorrhagic stroke, WHO sub-typing revealed a sensitivity of 86.3% and a specificity of 54.3% while Siriraj was found to have a sensitivity of 84.9% and specificity of 87.9%, showing that Siriraj is a better tool for stroke categorization for appropriate management in areas where neuroimaging is neither readily available nor affordable.



**Conclusion:** Stroke is a major cause of mortality and morbidity in North Central Nigeria. Community screening for risk factors should be pursued aggressively and identified risk factors managed promptly in order to reduce the burden of this pandemic. Siriraj stroke sub-typing can be used in resource limited setting like ours where neuroimaging facilities are either not available or too expensive.

## 5. How to choose different treatment modalities? A retrospective study of seizure outcome in resective epilepsy surgery, vagal nerve stimulator and ketogenic diet in paediatric refractory epilepsy and their underlying etiologies; sharing experience in Tuen Mun Hospital /a regional referral center in HKSAR

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*Tuen Mun Hospital*

**Aim:** To retrospectively study effectiveness of seizure control by resective epilepsy surgery, vagal nerve stimulator and ketogenic diet/modified Atkin diet in paediatric refractory epilepsy in Tuen Mun Hospital, HKSAR and what are the patients' underlying etiologies.

**Methods:** Children and adolescences with medical refractory epilepsy underwent resective epilepsy surgery, vagal nerve stimulator and ketogenic diet/modified Atkin diet in Tuen Mun Hospital were recruited to study seizure outcome and underlying etiologies. We use Engel Classification to measure seizure outcome after epilepsy surgery: the seizure outcome of KD/MAD categorized into groups as: (1) seizure free; (2) > 75% seizure reduction; (3) 50%-75% seizure reduction; and (4) seizure no change. The seizure outcome after vagal nerve stimulator categorized into groups as: (1) > 50% seizure reduction; and (2) Seizure no change.

**Results:** Forty-four patients underwent resective epilepsy surgeries from 2001 to July 2018. Age for surgery ranged from 0.8-19 years (mean 10.4 years). Follow-up duration ranged from 0.1-16 years (mean: 6.2 years) included 22 patients who underwent temporal lobe surgeries; 14 patients underwent extra-temporal lobe surgeries; 4 patients underwent hypothalamic hamartoma resection; 3 patients underwent disconnection or hemispherectomy. Seizure outcome in respective epilepsy surgery: in temporal lobe surgeries  $n = 22$ , Engel I: 82%; Engel II: 4%; Engel III: 8%; Engel IV: 8%. Etiologies: mesial temporal sclerosis: 41%; focal cortical dysplasia: 36%; developmental low grade tumor: 18%; gliosis: 5%. In extra-temporal lobe surgeries  $n = 15$ , Engel I: 64%; Engel III 36%. Etiologies: focal cortical dysplasia: 53%; cortical tuber 13%; ulgyria: 7%; haematoma/cavernous hamangioma: 13%, porencephaly: 7%; glial nodule: 7%. In hypothalamic hamartoma surgery, Engel I: 50%; Engel II: 25%; Engel IV: 25%. For disconnective surgery included 1 TPO disconnection & 2 hemispherotomy, Engel I: 33%; Engel II: 33%; Engel IV: 33%. Total 6 patients underwent vagal nerve stimulator implantation from 2014 to 2018. Three patients underwent re-implantation after VNS out of battery. Age of first implantation  $n = 6$  ranged from 3-22 years (mean age: 16.5 years). Duration of follow up: ranged 1-5.5 years (mean 3.7 years). The underlying etiologies included post-FIRES epilepsy, bilateral mesial temporal sclerosis, lennox gastaut syndrome, 2 subcortical band heterotopia, symptomatic epilepsy with history of status epilepticus due to TBC1D24 mutation. Three patients had > 50% seizure reduction with etiologies included post-FIRES epilepsy, LGS, TBC1D24 mutation. Three patients with no change in seizure control with etiologies 2 subcortical band heterotopia, bilateral MTS. Twenty-one patients put on ketogenic diet/ modified Atkin diet from 2001 to July 2018. Age to start KD/MAD ranged from 0.3 year to 11 years (mean age: 5 years). Duration of follow up ranged from 2 months to 232 months (mean 32 months). Three patients became seizure free with etiologies as congenital CMV infection, Landau Kleffner Syndrome,

suspected focal cortical dysplasia over TPO region in dominant hemisphere. Eight patients with > 75% seizure reduction with etiologies: Leigh's disease (complex I&IV respiratory chain enzyme deficiency), 2 suspected neuro-metabolic disease, post-HIE epilepsy, familial HLH, SSADH deficiency, post-influenza encephalopathy epilepsy, focal epilepsy. Five patients with 50%-75% with etiologies: Ohtahara's syndrome, post-HSV encephalitis, extensive right side fronto-central focal cortical dysplasia, 2 symptomatic generalised epilepsy. Five patients with seizure no change with etiologies: STXBP1 mutation, LGS, symptomatic epilepsy, 2 extensive focal/ hemi-cortical dysplasia, the latter two underwent epilepsy surgery with seizure improvement.

**Conclusion:** In view of better seizure outcome in surgery when compared with VNS and dietary treatment, refractory epilepsy patients with underlying lesion should consider epilepsy surgery. On the contrary, those with underlying metabolic or genetic disease and those with no definite focus or diffuse lesions should be considered for ketogenic diet or vagal nerve stimulator implantation.

## 6. Reminiscence therapy on enhancing cognitive and improving brain wave in mild cognitive impairment

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**Aim:** The aims of this research are to measure the effectiveness of reminiscence therapy on enhancing cognitive function and changes in brain waves in vascular MCI compared with MCI.

**Methods:** We used quasi-experimental research with pre-test and post-test study design, and statistical analysis F-test and  $\chi^2$ -test, with  $\alpha = 5\%$ . Subjects were divided into two groups, 16 in the MCI group and 16 in the MCI vascular group. Pre-tests were conducted prior to MOCA-INA and subjects' brain waves were examined with qEEG and brain mapping. Reminiscence therapies were conducted for 3 months for both groups and continued with the post-test.

**Results:** The result showed significant progress on cognitive enhancement with  $P$  value 0.0002 ( $P$  value < 0.05) both in the MCI group and MCI vascular group. The brain wave also showed some changes particularly in the MCI group; there were some significant changes in decreasing slow wave and increasing fast wave.

**Conclusion:** We can conclude that reminiscence therapy is effective on enhancing cognitive and improving brain wave for mild cognitive impairment.

## 7. Serum serotonin, a good indicator of insomnia in elderly with depression

**Retnaningsih**

*Diponegoro University/Dr. Kariadi General Hospital*

**Aim:** To evaluate the predictive value of serum serotonin for insomnia in elderly people.

**Methods:** Subjects in this retrospective study were 40 elderly people in Pucang Gading Nursing Home, Semarang, Central Java, Indonesia. Twenty-seven elderly people were diagnosed with insomnia. Serum serotonin level were measured in the elderly with and without insomnia. Bi-and multi-variate logistic regression were used to evaluate the impact of serotonin to predict insomnia.

**Results:** The median age of the whole population was 70 years and 90.0% were female. The elderly with serum serotonin level below 35.6 ng/mL have OR = 21.600 risk of developing insomnia compared with those with serum serotonin level more than 35.6 ng/mL ( $P = 0.000$ ). In bivariate analysis, low serotonin level was significantly associated with insomnia. In multivariate analysis, serotonin was an independent prognostic factor for insomnia ( $P = 0.001$ , OR = 0.046, 95% CI). The AUC for serotonin was 0.846 (95% CI = 0.708 s/d 0.958  $P < 0.001$ ).

**Conclusion:** We identified serum serotonin level predictive to insomnia in the elderly.

## 8. Chula Stroke self-help group: a tool for empowering stroke patient

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**Aim:** After acute stroke treatment in the hospital, long term post-stroke care is essential in order to prevent recurrent stroke and enhance quality of life. Stroke self-help groups engage patients, family members, and caregivers with the health care team including stroke physicians, psychiatrists, nurses, nutritionists, and social workers to help empower stroke survivors. The aim of this study is to evaluate the satisfaction among participants in the Chula Stroke Self-Help Group.

**Methods:** The Chula Stroke Self-Help Group has been established since 2016. The group meeting is held monthly at the outpatient department of King Chulalongkorn Memorial Hospital. The topic for each session is carefully selected by the nursing staff such as “Stroke is treatable”, “Stroke is preventable”, “Life after stroke”, and “Stroke Rehabilitation.” Each 90-min session is led by a stroke nurse, a social worker, and an invited speaker with 10-15 participants who were stroke patients, family members, or caregivers. The session begins with a brief introduction followed by extensive discussion among the participants to share their own experiences. After the session, the participants are asked to rate their satisfaction towards the activity.

**Results:** Thirty sessions were held between January 2016-June 2018 with a total of 351 participants. Among these, 220 (62.7%) participants were female. Most of the participants were stroke patients (49.8%), followed by family members of stroke patients (44.7%) and caregivers (5.4%). Most of the participants had a high level of satisfaction regarding self-empowerment (96.8%), knowledge (93.7%), and applicability to their situation (91.6%).

**Conclusion:** Stroke self-help group at King Chulalongkorn Memorial Hospital can be considered as a tool for empowering stroke patients, family members, and caregivers. Participants had high satisfaction toward the activity held by the self-help group.

## 9. Role of S100 $\beta$ protein as a prognostic factor in patient with acute intracerebral hemorrhage

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S100 $\beta$  protein has shown its potential as biomarker of brain injury, however its efficacy is less known in intracerebral hemorrhage (ICH) cases. Patients with ICH, who have same hematoma volume at the same location and received similar appropriate treatments, could have different neurological deficits and different prognoses. It is believed that many damages occurring in the subacute period were due to a complex pathophysiology pathway that still needs to be proven. There were 46 intracerebral hemorrhagic patients who entered the hospital consecutively during the period of August to December. CT scans were performed and met the inclusion and exclusion criteria. Neurological deficits as ascertained with National Institute of Health Stroke Scale (NIHSS). Blood samples were taken at admission to the hospital and 7th day of onset. There were 25 men (54.3%) with a median age of 56 (31-76) years. The most important risk factor was hypertension (78.3%). Median serum S100B levels in the ICH patients were significantly higher than those in normal individuals [22.70 (19.06-445.99) vs. 16.3 (15.58-23.79) pg/mL,  $P < 0.001$ ] and serum S100 $\beta$  protein level was significantly higher in the non-survival group than in the survival group [25.86 (19.78-445.99) vs. 21.325 (19.06-63.28) pg/mL,  $P = 0.032$ ]. Serum S100 $\beta$  protein levels correlated significantly with NIHSS ( $r = 0.418$ ;  $P = 0.004$ ). Area Under Curve (AUC) of S100 $\beta$  was  $0.839 \pm 0.103$  (95% CI, 0.638-1.000), cutoff level was 28.505 pg/mL with 80% sensitivity and 87.8% specificity ( $P = 0.014$ ). S100 $\beta$  protein levels correlated significantly with mortality within 1 week ( $P = 0.032$ ). High levels of S100 $\beta$  are present in the peripheral blood of patients with ICH and seem to correlate with neurological deficit and mortality within 1 week. Serum S100 $\beta$  level may be useful as marker for evaluating the prognosis of ICH.

## 10. Evaluation of language dysfunction and psychological state in stroke patients

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**Aim:** Evaluate multiple faculties of language function in about 116 patients with stroke in the past 8 months.

**Methods:** It's an observational study that evaluated all the stroke patients who were treated at ZH Sikder Institute of Neurosciences. Excluded the patients with other comorbidity that caused dementia and language dysfunction viz, PD, Cortico basal degeneration, Alzheimer's dementia.

**Results:** Most of the respondents were males (90%) ranging from 35 to 85 years. Most respondents had changeable risk factors. Significant language disturbance was seen in 30% of the patients and over 70% of the patients had minor impairment in language function. Emotional disturbance was seen in over 90% of these patients.

**Conclusion:** Language dysfunction is more common in stroke than we traditionally expect. Emotional

and psychological disturbance in association with language impairment make it challenging for clinicians to treat stroke patients. Understanding the problem can lead to early detection and multidisciplinary intervention, which might be the key to good post-stroke recovery.

## 11. Decrease in daily dose of mestinon, methylprednisolon and level of depression affected by self motivation therapy in myasthenia gravis patients

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**Aim:** Myasthenia gravis is an autoimmune disease<sup>[1]</sup>. One of etiologies of myasthenic crisis is emotional and physical stress<sup>[2]</sup>. We examined depression levels using the Hamilton Depression Rating Scale<sup>[3]</sup> and the daily dose of mestinon and methylprednisolone<sup>[4]</sup> in myasthenia gravis patients before and after 1 month of self motivation therapy.

**Methods:** Myasthenia gravis patients including 7 males and 18 females at Semarang Myasthenic Society were measured the level of depression with Hamilton Depression Rating Scale, daily dose of mestinon and methylprednisolon before and after 1 month self motivation therapy through motivational posters ("Thank God, I am alive and happy. Physical and emotional stress will only cause relapse of myasthenia, avoid!") that were posted in all rooms where patients could read them every day. The relationship between Hamilton Depression Rating Scale and drug dose were evaluated by using the SPSS 19.0 software. Chi square test was conducted to evaluate the categorical data. The compatibility of variables to the normal distribution was reviewed through Shapiro Wilk test. Student's t test and Wilcoxon test were used to evaluate the parametric and non parametric data. The significance limit was considered to be  $P < 0.05$ .

**Results:** Hamilton score depression scale before treatment ranged from 9 to 19, average as mild to moderate depression ( $13.24 \pm 2.17$ ). After being given self motivation therapy for 1 month there was a change in the hamiltonton depression scale score range from 2 to 9, on average relatively normal ( $4.84 \pm 1.82$ ). There was no myasthenic crisis that occurred during the 1-month motivation period. There is a change in the dose of mestinon (60 mg) daily, before therapy 2-4 times a day while post therapy 2-3 times a day, and methylprednisolone (4 mg) daily, methylprednisolone once a day, while on therapy not used at all. Self motivational therapy has a significant effect on the Hamilton Depression Rating Scale score, changes in mestinon and methylprednisolone daily dose with  $P < 0.001$ . Changes in depression levels affect the daily dose of mestinon ( $P = 0.038$ ) but does not affect methylprednisolone ( $P = 0.371$ ).

**Conclusion:** Reduced stress levels through self-acceptance and gratitude for life affect the level of depression and the daily dose of mestinon in myasthenia gravis patients.

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## 12. Life quality and the self in severe dementia: an intervention

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The goal of psychosocial interventions in dementia is to improve the quality of life (QOL) of the sufferer. Dementia is a multifaceted disorder that affects many aspects of life and of the self. An effective intervention could target all aspects of the self, and of the disorder. The ultimate goal is to improve the QOL of the person, through enhancing the self, and all its aspects. This is challenging in severe dementia.

An intervention took place that aimed at enhancing all domains of the self, in order to improve QOL. Participants were 3 females and 2 males. All experienced individual sessions, three times per week for two months, with activities using all arts, while focusing on existing skills. The activities had a cognitive component, an emotional (expressive) one, a behavioral, and physical engagement.

These individuals had severe dementia, and no family caregivers, making assessment challenging. In order to detect the self, the I-AM test was used (requiring the completion of ten sentences starting with “I am...”), and to detect QOL, the QUALID Scale was used- an observational measure completed by the researcher and by professional caregivers.

The assessment indicated an improvement in the sense of self, and in QOL for all participants. This result can be explained psychotherapeutically and neuroscientifically: participants were brought to the “here and now” and their existing skills (and self) were enhanced, making them more functional; thus, improving their QOL, while several brain functions were combined and engaged in each session, promoting the delaying of the disorder.

Several implications arise: the self is maintained in dementia, even in the latest stage, and could be targeted in treatment, while it appears to be linked with QOL. Finally, there is a need for an intervention in severe dementia, a need to improve the living of the sufferers, and a need to focus on the abilities that remain.



Original Article

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# Predictors of severity and outcome and roles of intravenous thrombolysis and biomarkers in first ischemic stroke

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## ABSTRACT

**Aim:** Stroke is one of the leading causes of death and disability. The proportion of patients receiving recombinant tissue plasminogen activator is low in our country. Biomarkers to identify patients at risk of severe disease, and guide treatment and prognosis would be valuable. This article aims to identify the factors that can independently prognosticate the acute phase of ischemic stroke.

**Methods:** All patients with the first episode of ischemic stroke admitted to the Neurology Department between 1st December 2017 to 31st March 2018 were included in this pilot study. Stroke severity was evaluated using the National Institute of Health Stroke Scale (NIHSS). Patients being admitted within 4.5 h of onset of symptoms were thrombolysed with injection alteplase. For each patient, 4 serum biomarkers (D-dimer, fibrinogen, C-reactive protein and neuron specific enolase) were evaluated at admission and 24 h later. Discharged patients were assessed on an outpatient basis using the modified Rankin scale. The study primarily aimed to identify the factors predicting the severity and outcome of stroke, and to evaluate the effect of thrombolysis on the outcome. The secondary aim was to evaluate the role of biomarkers to predict the unfavorable outcome and the chance of post thrombolysis hemorrhage.

**Results:** Out of 30 patients included in the study, 10 had NIHSS 0-4, 12 had NIHSS 5-15 and 8 had NIHSS 16-42. Sixteen patients had unfavorable outcome (mRS score  $\geq 2$ ), and 5 patients expired. Old age, history of diabetes, CHADS2 score  $\geq 2$ , and total anterior circulation stroke (TACS) independently affected stroke severity, whereas low



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ejection fraction < 35%, and TACS, independently predicted unfavorable outcome and mortality. High mean arterial blood pressure (MABP) and capillary blood glucose (CBG) at admission were significant predictors of stroke severity, unfavorable outcome, and mortality. Out of 10 thrombolysed patients, two had mRS score  $\geq 2$  and 3 had the post-thrombolysis hemorrhage. Thrombolysis significantly reduced the incidence of the unfavorable outcome, but did not significantly affect death. All the biomarker levels at admission were significantly higher among patients with severe stroke and those who subsequently had an unfavorable outcome. D-dimer levels significantly increased and fibrinogen level significantly decreased following thrombolysis. Higher MABP, CBG, and fibrinogen levels at admission predicted significantly higher chance to develop hemorrhagic complications post thrombolysis.

**Conclusion:** Low ejection fraction, occurrence of TACS and the higher levels of the biomarkers under study predicted poor outcome. Higher mean CBG and MABP and raised fibrinogen levels predicted higher chance of post-thrombolysis hemorrhage.

**Keywords:** First-ever ischemic stroke, thrombolysis, biomarkers, total anterior circulation stroke, fibrinogen

## INTRODUCTION

Stroke is one of the leading causes of death and disability in India, which is facing the double burden of communicable and non-communicable diseases. The estimated adjusted prevalence rate of stroke range from 84-262/100,000 in rural to 334-424/100,000 in urban areas. The incidence rate is 119-145/100,000 based on the recent population based studies. These values were higher than those of high-income countries<sup>[1]</sup>. Case fatality rates vary widely, the highest being 42% in Kolkata<sup>[2]</sup>. Among patients presenting with the first-ever stroke in the Mumbai registry, 80.2% were ischemic strokes and 17.7% were hemorrhagic strokes<sup>[3]</sup>. In the Trivandrum registry, 83.6% were ischemic strokes, 11.6% were intracerebral hemorrhages, and 4.8% were subarachnoid hemorrhages<sup>[4]</sup>. Thirty-two percent of the patients in the Kolkata registry had hemorrhagic stroke, the highest reported so far from India<sup>[5]</sup>. The proportion of patients receiving recombinant tissue plasminogen activator (rtPA) is low in our country, being 11% (104 out of 967 patients) in the on-going Indo USA National stroke registry, due to lack of trained personnel. Intraarterial and mechanical thrombolysis was given in 3.5% (34 out of 967 patients)<sup>[6]</sup>.

Age, stroke severity, stroke mechanism, infarct location, comorbid conditions, clinical findings, and related complications influence stroke prognosis. Interventions such as thrombolysis, stroke unit care, and rehabilitation also influence the outcome of ischemic stroke. Knowledge of these prognostic factors enables the clinician to make a reasonable prediction for each patient, to offer a rational treatment to the patients, and to help the family members understand the disease course<sup>[7]</sup>. Though clinical examination can excellently assess the stroke patients and the disease progression, biomarkers would be valuable to identify the patients at risk of severe disease, to guide treatment and to reasonably predict the prognosis. Though many such proteins which are markers of brain tissue damage, inflammation, and coagulation/thrombosis are associated with ischemic stroke, their successful translation to a biomarker useful in clinical practice has proven difficult due to the heterogeneity of ischemic stroke<sup>[8]</sup>. Moreover, they are not specific to ischemic stroke, as many other disease processes can damage brain tissue. The blood-brain barrier restrains the release of these biomarkers into the systemic circulation; hence, their levels may not correlate with the infarct volume, given that the anatomic locations of stroke have different impacts on blood-brain barrier breakdown<sup>[9]</sup>. Markers of ischemic brain injury include S100 calcium binding protein B (S-100B), neuron-specific enolase (NSE), myelin basic protein, and glial fibrillary acidic protein. Several proteins involved in inflammation and immune response have also been identified as biomarkers of ischemic stroke, including C-reactive protein (CRP), interleukin-6, tissue necrosis factor-alpha, vascular cell adhesion protein 1, intercellular adhesion molecule 1, N-methyl-d-aspartate receptor antibodies and matrix metalloproteinases. Similarly, molecules involved in

acute thrombosis have also been associated with ischemic stroke, including fibrinogen, D-Dimer and von-Willebrand factor<sup>[10]</sup>.

Few studies have systematically evaluated the multimodal factors (demographic, clinical, radiological and biological markers) in unselected consecutive first-ever ischemic stroke patients. This article aims to identify the factors that can independently prognosticate the acute phase of ischemic stroke.

## METHODS

All patients with the first episode of ischemic stroke admitted to the Neurology Department between 1st December 2017 to 30th April 2018 were included in this pilot study and written informed consent was taken from the patients or their family members to participate in the study. Stroke was defined according to the World Health Organisation criteria<sup>[11]</sup>. Ischemic stroke was diagnosed with a combination of clinical criteria and non contrast computed tomography imaging of brain, which was done for all patients upon admission, to exclude intracerebral hemorrhage. We recorded the medical history prior to the stroke and the congestive heart failure, hypertension, age, diabetes mellitus, prior stroke or transient ischemic attack (TIA), or thromboembolism (CHADS<sub>2</sub>) scores were calculated for all patients. The following variables were analyzed: gender, age, domestic arrangements (lives with other family members or alone), clinical history, and medications, vascular risk factors including history of hypertension, diabetes mellitus, heart diseases [ischemic heart disease, low ejection fraction and atrial fibrillation (AF), as a history of AF and/or AF diagnosed during the index admission by electrocardiography], TIA, current or former smoking, and hypercholesterolemia. Stroke severity was evaluated in the acute phase of the initial stroke by a neurologist certified in the use of the National Institute of Health Stroke Scale (NIHSS). Stroke severity by NIHSS<sup>[12]</sup> was categorized as mild (0-4), moderate (5-15), or severe (16-42). Furthermore, strokes were classified according to the Bamford criteria<sup>[13]</sup> in total anterior circulation stroke (TACS), partial anterior circulation stroke (PACS), posterior circulation stroke (PCS), and lacunar stroke (LS). Patients being admitted within 4.5 h of onset of symptoms were thrombolysed with injection alteplase, provided they did not have the contraindications for thrombolysis. For each patient, 4 serum biomarkers [(D-dimer, fibrinogen, CRP and Neuron specific enolase (NSE))] were evaluated at admission and 24 h later. D-dimer was assessed using enzyme linked immunosorbant assay (ELISA) kits from GenWay Biotech, San Diego, California, USA, and a level of more than 4 µg/mL was considered high. NSE was assessed using human NSE ELISA kit (Elabscience Biotechnology Co. Ltd., Houston, USA) and the normal value at spectrophotometers in the 450 nm wavelength was 7.2-12 ng/mL. Serum concentrations of CRP were quantified using a commercially available turbidimetric immunoassay (Transasia Bio-Medicals Ltd., Erba Diagnostics, Mannheim, Germany) and value < 6 mg/L was considered normal. Fibrinogen was assayed by FibroTek fibrinogen kit (R2 Hemostasis Diagnostics India Private Limited, Indira Puram, Ghaziabad, Uttar Pradesh) and the value of 150-400 mg/dL was considered normal.

Follow-up magnetic resonance imaging examination (1.5 Tesla system providing axial T1, T2, and proton density weighted images) or brain CT scan was repeated 5 days after the index event. Metabolic profile (renal and liver function tests); and hematologic parameters (complete hemograms and coagulation profile) were recorded in the registry on arrival and again at 24-48 h after stroke onset. The cardiological profile (electrocardiogram and transthoracic echocardiography) and a search for vasculitis (antinuclear factor and antiphospholipid antibodies) were also done. Previous infections were excluded by medical history, chest radiograph, routine urinalysis, and complete physical examination. The vital parameters were recorded continuously using multi-parameter monitors. The length of hospital stay was defined from the day of admission to a hospital ward to the day of discharge. Acute stroke management and secondary prevention in these patient followed current European Stroke Organization guidelines<sup>[14]</sup>. Discharged patients were followed up on a monthly basis through neurological examination and review of records. Their clinical outcomes were assessed using the modified Rankin scale (mRS) and categorized as favorable (score 0-1) or unfavorable (score 2-6). Exclusion criteria included history of recent infection, obvious signs of acquired infection before stroke

onset, and an initial CRP level > 10 mg/dL due to presumed infection.

Outcome measures - the primary aims of the study were the following: (1) to identify the factors independently predicting: i) the severity of stroke; ii) the unfavorable outcome at 30 days post discharge; iii) the mortality; and iv) the chance of post thrombolysis hemorrhage; and (2) to evaluate the effect of thrombolysis on the outcome of ischemic stroke patients. The secondary aim was to evaluate the role of biomarkers to predict the unfavorable outcome and the chance of post thrombolysis hemorrhage and to evaluate the change of biomarker levels post thrombolysis.

The patient characteristics, comorbid risk factors, and hospital investigations were assessed by Chi-square test for categorical variables and independent-samples *t* test and one way analysis of variance for continuous variables. The variables analyzed were age, gender, body mass index, life conditions, comorbidities, NIHSS at admission, vascular risk factors, therapy prior to stroke, addictions, pathophysiologic and metabolic factors. Multivariate logistic regression models estimated the impact of possible determinants of stroke severity at admission. Differences between groups and effect of patient characteristics on the clinical outcome were assessed using Chi-square test. Statistical tests were considered significant when the value was  $\leq 0.05$ . Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 20.0 software version.

## RESULTS

Out of 30 patients admitted with first ischemic stroke, 15 patients arrived at the hospital within 4.5 h. Out of them, 2 patients had NIHSS score > 25, 2 patients had stroke involving > 1/3 cerebral hemisphere, and 1 patient was taking oral anticoagulants for dilated cardiomyopathy - hence they did not receive thrombolysis. The rest 10 patients were thrombolysed with injection alteplase. The mean time (standard deviation or SD) of presentation of patients who were thrombolysed was 3.8 (0.7) and 7.8 (2.4) h for the remaining patients. Twelve patients had a mild stroke, 10 had a moderate stroke and 8 had a severe stroke. Fourteen patients had mRS score < 2, and 16 had mRS score  $\geq 2$ , among whom 5 patients expired. Three out of ten thrombolysed patients developed intracerebral hemorrhage, among whom 1 patient expired. During admission, all the cases of AF were already diagnosed and were on anticoagulants as per the current guidelines. The results have been described in [Tables 1-6](#).

## DISCUSSION

### Discussion on determinants of stroke severity and outcome

In this study on first-ever ischemic stroke patients, we demonstrated that risk factors such as old age, history of diabetes, CHADS<sub>2</sub> score  $\geq 2$ , and TACS independently affected stroke severity, whereas low EF < 35%, and TACS, independently predicted the unfavorable outcome (mRS score  $\geq 2$ ) and mortality. High mean arterial blood pressure (MABP) and capillary blood glucose (CBG) at admission were significant predictors for stroke severity, mRS score  $\geq 2$  and mortality. Female patients had significantly higher incidence of unfavorable outcome, but female gender was not a significant predictor of stroke severity and mortality. CHADS<sub>2</sub> score significantly predicted the unfavorable outcome, but it was not a significant predictor when mortality was considered alone. Hypertension, hypercholesterolemia, smoking, ischemic heart disease, and AF showed a non-significant trend to be more prevalent among patients with severe stroke, unfavorable outcome and mortality. History of TIA was significantly associated with higher incidence of severe stroke and mortality. Home medications, living conditions, PACS and PCS did not significantly contribute to the severity and outcome of the stroke. However, patients of LS had a significantly lesser risk of having an unfavorable outcome. In the previous population-based studies, old age was found to be a strong independent predictor of ischemic stroke severity, outcome and mortality<sup>[7,15]</sup>. In Corso's study<sup>[7]</sup>, patients > 85 years of age had 2.9 times higher risk for having a severe stroke. In our study, out of 3 patients > 80 years of age, 2 had a severe stroke [odds ratio (OR) = 7 (0.53-91.11), *P* = 0.16]. Previous reviews reported that female gender

**Table 1. Patient characteristics at time of initial stroke**

Characteristics	Mild stroke (n = 12)	Moderate stroke (n = 10)	Severe stroke (n = 8)	P value
Female sex	5	6	3	1.0
BMI (kg/m <sup>2</sup> )	25.3 (0.3)	26.5 (0.5)	26.9 (0.8)	0.045
Age	58.2 (6.2)	62.4 (10.8)	66.3 (6.3)	0.01
Living condition				
Lives alone	2	1	3	0.6
Lives with family	10	9	5	
Vascular risk factors				
Hypertension	8	9	8	0.11
Hypercholesterolemia	8	7	7	0.6
Diabetes	3	8	7	0.02
Previous TIA	0	2	3	0.04
Current smoking	5	4	3	1.0
Ischemic heart disease	1	1	2	0.53
AF	1	2	2	0.53
Low EF (< 35%)	1	2	3	0.25
CHADS2 score				
0-1	10	1	2	
≥ 2	2	9	6	0.01
Bamford classification				
TACS	0	3	4	0.014
PACS	3	2	1	0.6
PCS	2	3	3	0.6
LS	7	2	0	0.14
MABP at admission	105.4 (8.8)	112 (9.2)	116 (7.8)	0.013
CBG at admission	152.1 (8.2)	163.7 (11.8)	181.4 (10.1)	< 0.0001
Home medications				
Statins	5	5	2	0.64
Antihypertensives	6	5	3	0.4
Anticoagulants	1	2	2	0.5

P value-statistical analysis was performed on the NIHSS 0-4 and the NIHSS ≥ 16 groups. The univariate analysis in Table 1 identified that higher BMI and age, diabetes, previous TIA, CHADS2 score ≥ 2, occurrences of TACS, higher MABP and CBG at admission significantly contributed to the occurrence of severe stroke. Multiple logistic regression analyses identified that the presence of diabetes [adjusted OR, i.e., AOR (95% CI) = 16.20 (2.5-180.45), *P* = 0.025], CHADS2 ≥ 2 score [AOR = 14.8 (1.2-130.17), *P* = 0.02, and occurrence of TACS (AOR = infinity, *P* = 0.016) independently influenced the higher NIHSS scores (≥ 16). Six patients had cardioembolic stroke. EF: ejection fraction; MABP: mean arterial blood pressure; CBG: capillary blood glucose; BMI: body mass index; TIA: transient ischemic attack; TACS: total anterior circulation stroke; PCS: posterior circulation stroke; LS: lacunar stroke; AOR: adjusted odds ratio

**Table 2. Results of multivariate logistic regression analysis model for probability of unfavorable outcome (mRS score ≥ 2)**

Characteristics	mRS score ≥ 2 (n = 16)	OR (95% CI)	RR (95% CI)	P value
Female sex (n = 14)	11	0.1636 (0.03-0.83)	0.47 (0.23-0.95)	0.032
Hypertension	15	6 (0.58-61.8)	1.3 (0.92-1.8)	0.15
Hypercholesterolemia	12	1.2 (0.23-6.0)	1.05 (0.67-1.6)	1.0
Diabetes	10	1.25 (0.2-5.4)	1.09 (0.6-1.97)	1.0
Previous TIA	4	4.33 (0.4-44.4)	3.5 (0.44-27.7)	0.33
Current smoking	9	4.7 (0.9-23.6)	2.6 (0.88-7.8)	0.07
Ischemic heart disease		0.85 (0.1-7.4)	0.87 (0.14-5.4)	1.0
AF	4	4.33 (0.4-44.4)	3.5 (0.44-27.7)	0.33
Low EF (< 35%)	6			0.018
CHADS2 score ≥ 2	13	10.83 (1.9-59.8)	2.84 (1.2-6.7)	0.008
TACS	7			0.007
PACS	3	0.84 (1.4-5.07)	0.87 (0.2-3.65)	1
PCS	6	3.6 (0.59-21.9)	2.6 (0.62-10.9)	0.22
LS	2	0.14 (0.02-0.8)	0.25 (0.06-1.01)	0.045

The mean (SD) age and BMI among patients with mRS score ≥ 2 and mRS score < 2 were 68.6 (7.4) years vs. 62.7 (8.2) years (*P* = 0.047) and 26.3 (0.7) kg/m<sup>2</sup> vs. 25.8 (0.5) kg/m<sup>2</sup> (*P* = 0.043). At admission, the mean (SD) MABPs and CBGs among patients with mRS score ≥ 2 and mRS score < 2 were 115.5 (5.9) mm of Hg vs. 110.4 (4.6) mm of Hg (*P* = 0.025) and 186.6 (29.8) mg/dL vs. 150.4 (14.6) mg/dL (*P* = 0.0003). Thus, the univariate analysis in Table 2 identified female sex, smoking, low EF, CHADS2 score ≥ 2, the occurrence of TACS, higher age and BMI and higher MABP and CBG at admission as predictors of mRS score ≥ 2. Patients with lacunar stroke had a significantly lower incidence of mRS score ≥ 2 (*P* = 0.045). Multivariate analysis identified low EF (AOR = infinity), CHADS2 score ≥ 2 [AOR = 11.1 (2.1-54.2), *P* = 0.009], and occurrence of TACS (AOR = infinity) to be independent predictors of mRS score ≥ 2. EF: ejection fraction; TACS: total anterior circulation stroke; PACS: partial anterior circulation stroke; PCS: posterior circulation stroke; LS: lacunar stroke

**Table 3. Results of multivariate logistic regression analysis model for probability of mortality**

Characteristics	Mortality (n = 5)	OR (95% CI)	RR (95% CI)	P value
Female sex (n = 14)	3	1.9 (0.27-13.49)	1.7 (0.33-8.83)	0.64
Hypertension	4	0.76 (0.06-8.7)	0.8 (0.11-5.7)	1.0
Hypercholesterolemia	4	1.55 (0.14-16.4)	1.45 (0.18-11.14)	1.0
Diabetes	3	1.55 (0.14-16.4)	1.45 (0.18-11.14)	1.0
Previous TIA	3	17.25 (1.72-172.02)	7.5 (1.65-33.94)	0.02
Current smoking	2	1 (0.14-7.69)	1 (0.19-5.12)	1
Ischemic heart disease	2	7.66 (0.76-76.45)	5 (0.9-27.06)	0.118
AF	2	4.88 (0.96-42.3)	3.33 (0.73-15.08)	0.18
Low EF (< 35%)	3	7.33 (1.27-95.18)	4.16 (1.1-15.7)	0.041
CHADS2 score ≥ 2	4	3.69 (0.36-37.85)	1.53 (0.86-2.74)	0.35
TACS	4	29.33 (2.4-357.86)	6.66 (2.11-21.02)	0.0057
PACS	1	1 (0.09-11.02)	1 (1.39-6.8)	1
PCS	3	6 (0.78-46.14)	3 (1.03-8.67)	0.1
LS	0			0.28

The mean (SD) age of expired patients was 66.5 (7.4) years compared to 60.7 (9.3) years for surviving patients ( $P = 0.201$ ), and mean BMI of expired patients was 25.4 (0.8) kg/m<sup>2</sup>, compared to 24.9 (0.7) kg/m<sup>2</sup> for surviving patients ( $P = 0.16$ ). At admission, the mean (SD) MABP among survivors was 115.2 (5.9) mm of Hg vs. 110.3 (4.2) mm of Hg among expired patients ( $P = 0.033$ ). The mean (SD) CBGs at admission were 179.9 (12.5) mg/dL among survivors vs. 152.6 (20.6) mg/dL among expired patients ( $P = 0.0001$ ). The univariate analysis in Table 3 identified previous TIA, low EF, the occurrence of TACS, higher age and BMI and higher MABP and CBG at admission as predictors of mortality. Multivariate analysis identified low EF [AOR = 7.21 (1.5-90.21),  $P = 0.043$ ], and the occurrence of TACS [AOR = 28.9 (3.5-320.47),  $P = 0.006$ ] to be independent predictors of mortality. TIA: transient ischemic attack; EF: ejection fraction; TACS: total anterior circulation stroke; PACS: partial anterior circulation stroke; PCS: posterior circulation stroke; LS: lacunar stroke

**Table 4. Mean (SD) levels of biomarkers at admission among different groups of stroke patients**

Biomarker	Mild stroke (n = 12)	Moderate stroke (n = 10)	Severe stroke (n = 8)	P value	mRS score < 2 (n = 14)	mRS score ≥ 2 (n = 16)	P value
CRP (mg/L)	4.7 (1.4)	5.7 (2.3)	6.9 (3.1)	0.043	5.6 (0.9)	6.9 (2.1)	0.040
Fibrinogen (mg/dL)	390.67 (20.25)	456.4 (40.50)	500.75 (37.86)	< 0.0001	430 (29.75)	478 (37.65)	0.0007
D-dimer (μg/mL)	4.7 (0.5)	6.4 (2.2)	8.8 (2.5)	< 0.0001	5.6 (1.4)	7.2 (2.5)	0.043
NSE (ng/mL)	24.5 (5.4)	37 (11.9)	56 (20.5)	< 0.0001	30.6 (6.8)	47 (16.87)	0.0021

Table 4 demonstrated that all the biomarker levels at admission were significantly higher among patients with severe stroke and unfavorable outcome (statistical analysis was performed on the mild and severe stroke groups). CRP: C-reactive protein; NSE: neuron specific enolase

**Table 5. Comparison of final outcome and mean (SD) levels of the biomarkers 24 h after admission among thrombolysed vs. non-thrombolysed patients**

Characteristics	Thrombolysed (n = 10)	Non- thrombolysed (n = 20)	P value
mRS ≥ 2 (n = 16)	2 (20%)	14 (70%)	0.018
Death (n = 5)	1 (10%)	4 (20%)	0.64
CRP	5.32 (0.9)	5.87 (2.1)	0.43
Fibrinogen	420 (20.5)	479.2 (29.4)	< 0.0001
D-dimer	6.6 (3.0)	4.9 (2.0)	0.047
NSE	36.5 (14.6)	40.2 (16.8)	0.54

Table 5 showed that thrombolysis significantly reduced the incidence of the unfavorable outcome, but did not significantly affect death. Three patients with TACS, two with PACS, three with PCS and two with LS underwent thrombolysis. One each with TACS and PCS had mRS ≥ 2. Thus, there was no significant difference in the efficacy of thrombolysis on stroke in various arterial territories ( $P > 0.05$ ). D-dimer levels significantly increased and fibrinogen level significantly decreased following thrombolysis. CRP: C-reactive protein; NSE: neuron specific enolase; TACS: total anterior circulation stroke; PACS: partial anterior circulation stroke; LS: lacunar stroke.

has more severe strokes than men, with a 1-month case fatality of 24.7% vs. 19.7% for males<sup>[16]</sup>. The case fatality rates were lower in our study - 21.4% among females and 12.5 % among males. In Corso's study, the female patients were older and suffered more frequently from AF<sup>[17]</sup>; hence, females had a more severe stroke. Previous studies have reported that stroke patients with AF mostly present with large cortical infarcts, and less frequently with lacunar infarcts compared with patients without AF due to the lack of collateral vessels,



**Table 6. Comparison of the baseline characteristics and biomarker levels at admission for patients developing hemorrhage (hge.) post thrombolysis**

Characteristics	Patients with hge. complications (n = 3)	Patients without hge. complications (n = 7)	P value
BMI (kg/m <sup>2</sup> )	26.5 (1.1)	26.8 (0.9)	0.66
Mean age (SD)	64 (10.8)	63 (8.2)	1.0
Vascular risk factors			
Hypertension	3	4	0.475
Hypercholesterolemia	3	4	0.475
Diabetes	2	3	1.0
AF	1	2	1.0
CHADS2 score			
≥ 2	1	3	1.0
Bamford classification			
TACS	2	2	0.5
PACS	0	3	0.475
PCS	1	2	1.0
MABP at admission	110.5 (4.2)	118.8 (4.8)	0.032
CBG at admission	251.4 (109.4)	143.2 (45.7)	0.048
Mean NIHSS at admission (SD)	10.4 (5.8)	20.2 (4.0)	0.0137
CRP (mg/L)	5.88 (0.24)	5.64 (0.32)	0.28
Fibrinogen (mg/dL)	478.5 (20.85)	421.4 (26.4)	0.01
D-dimer (μg/mL)	6.88 (1.4)	6.38 (1.7)	0.5
NSE (ng/mL)	42.1 (11.2)	30.8 (4.8)	0.07

Table 6 demonstrated that higher MABP, CBG, and fibrinogen levels at admission predicted significantly higher chance to develop post-thrombolysis hemorrhagic complications. Other three biomarkers were not significant predictors of hemorrhagic complications. (N.B- in Tables 1, 2, 3, and 6 some patients had multiple comorbidities). BMI: body mass index; TACS: total anterior circulation stroke; PACS: partial anterior circulation stroke; PCS: posterior circulation stroke; MABP: mean arterial blood pressure; CBG: capillary blood glucose; CRP: C-reactive protein; NSE: neuron specific enolase.

which develop and compensate for acute arterial occlusion in patients with gradual occlusion of arteries, such as in atherosclerosis of cervical or cerebral arteries<sup>[18]</sup>. Steger *et al.*<sup>[19]</sup> reported that for AF patients with ischemic stroke, the in-hospital mortality was higher (25% vs. 14%,  $P < 0.0004$ ) and neurological outcome was poorer (65 vs. 90 Barthel index,  $P < 0.0004$ ). But in our study, multivariate analysis did not establish AF as an independent predictor of mortality. AF was non-significantly more frequent among patients with severe stroke, unfavorable outcome, and among those who expired. Ntaios *et al.*<sup>[20]</sup> reported that compared with CHADS2 score 0, patients with CHADS2 score 1 and CHADS2 score  $> 1$  had higher risks of ischemic stroke [hazard ratio 2.38 (95% CI: 1.41-4.00) and 2.72 (95% CI: 1.68-4.40), respectively] and death [hazard ratio 3.58; (95% CI: 1.80-7.12), and 5.45 (95% CI: 2.86-10.40) respectively]. In our study, CHADS2 score  $\geq 2$  significantly predicted stroke severity but did not predict mortality. Di Tullio *et al.*<sup>[21]</sup> in their “Reduced Ejection Fraction Trial”, demonstrated that baseline left ventricular EF  $< 15\%$  was inversely and linearly associated with the primary outcome, and mortality. Even in warfarin-treated patients, each 5% EF decrement significantly increased the stroke risk [adjusted hazard ratio 2.125 (95% CI: 1.182-3.818)]. In our study, EF  $< 35\%$  [mean (SD) EF = 26.8 (5.8)] significantly predicted unfavorable outcome and mortality. Although Nedeltchev *et al.*<sup>[22]</sup> and Musolino *et al.*<sup>[23]</sup> reported current smoking, followed by hypercholesterolemia, family history of cerebrovascular disease, and hypertension to be the most prevalent risk factors among young ischemic stroke patients, these factors were non-significant predictors of stroke severity and outcome in our study. Osmani *et al.*<sup>[24]</sup> reported that TACS had the worst outcome with the highest number of mortalities (72.2%). The LS had a better outcome, i.e. 65.7% of the patients were functionally independent by the end of 6 months compared to 15% of TACS patients<sup>[24]</sup>. In our study, patients with TACS had a significantly higher incidence of severe stroke (57.14%), unfavorable outcome (100%) and mortality (57.14%). Huang *et al.*<sup>[25]</sup> also described that TACS was associated with a poor functional outcome, but patients had a better outcome with LS. Medications use like statins, anticoagulants and antihypertensives did not affect stroke prognosis, similar to Corso’s study<sup>[7]</sup>. Koton *et al.*<sup>[26]</sup> described that systolic blood pressure (SBP) at admission was associated with stroke severity and disability at discharge or in-hospital death with an adjusted OR of 1.06 (95% CI: 1.04-1.08)

per 10mmHg change in SBP. In our study too, the higher MABP at admission was significantly associated with the stroke severity, the unfavorable outcome, and mortality. Bruno *et al.*<sup>[27]</sup> described that in all strokes combined ( $P = 0.03$ ) and in non-LSs ( $P = 0.02$ ), higher admission blood glucose levels were associated with the worse outcomes at 3 months according to multivariate logistic regression analysis adjusted for stroke severity, diabetes mellitus, and other vascular risks, thereby, corroborating with our findings.

### Discussion on the role of thrombolysis

Our study demonstrated that thrombolysis significantly reduced the incidence of the unfavorable outcome, which is evidenced by the fact that only 20% of thrombolysed patients and 70% of non-thrombolysed patients had the unfavorable outcome. Mehta *et al.*<sup>[28]</sup> reported 67.1% good outcome and 32.9% unfavorable outcome post thrombolysis; diabetes, dyslipidemia, NIHSS at admission  $> 15$ , blood sugar  $> 250$  mg/dL, dense cerebral artery sign and occlusion of large artery being significant predictors of the poor outcomes on multivariate analysis. Liu *et al.*<sup>[29]</sup> reported that age  $\geq 70$  years, NIHSS score  $> 20$ , serum glucose on admission  $> 9.0$  mmol/L and cardioembolism were independent predictors of hemorrhage after thrombolysis in Chinese patients with acute ischemic stroke. Thirty percent patients in our study had post-thrombolysis hemorrhage. Blood glucose, MABP, higher mean NIHSS score and serum fibrinogen level at admission correlated significantly with post-thrombolysis hemorrhage. Higher NIHSS score increases the risk of hemorrhages since severe ischemic stroke is reflected by large areas of injured brain tissue, including injured blood vessels, which are prone to bleeding after rtPA treatment<sup>[30]</sup>.

### Discussion on the role of biomarkers

Our study demonstrated that the mean values of all the 4 biomarkers-CRP, fibrinogen, D-dimer, and NSE were significantly higher among patients with severe stroke. Fibrinogen level decreased and D-dimer level increased significantly following thrombolysis. Fibrinogen was the only biomarker, whose elevated levels could significantly predict post-thrombolysis hemorrhage. Results of a meta-analysis<sup>[31]</sup> indicated a significant association between the elevated baseline CRP and unfavorable long-term functional outcome. Although our study is based on the CRP values at admission, interestingly, two studies of the meta-analysis<sup>[32,33]</sup> showed a stronger association of poor outcome with hs-CRP measurements at 24-48 h and 7 days reflecting impairment of the recovery process due to prolonged inflammation after ischemic stroke.

Rothwell *et al.*<sup>[34]</sup> described that fibrinogen predicted ischemic stroke, with the association tending to be stronger in patients with nonlacunar than lacunar syndromes. Moreover, fibrinogen levels were found to be an independent predictor of early neurological deterioration among diabetic patients<sup>[35]</sup>. The Alteplase-Tenecteplase Trial Evaluation for Stroke Thrombolysis study demonstrated that alteplase was associated with prolongation of prothrombin time, reduced fibrinogen and plasminogen, elevated fibrin degradation products and d-dimer level<sup>[36]</sup>. Following ischemic stroke, tissue responds with mitochondrial dysfunction and increased nitric oxide (NO) production which vasodilates and maintains blood perfusion. In turn this leads to a burst in free radical production and the generation of peroxynitrite, which irreversibly nitrates proteins. Fibrinogen's up-regulation as an acute-phase reactive protein and increased permeability of the blood-brain barrier during ischemic stroke allowing extravasation of different plasma proteins into the brain parenchyma further potentiates fibrinogen nitrotyrosination. At early stages nitro-fibrinogen delays clot formation, but in the long term, it becomes harmful due to the production of fibrinolysis resistant clots and the induction of neuronal damage<sup>[37]</sup>. Hence, possibly more elevation in the fibrinogen level produces more cellular and local vascular damage, resulting in a higher chance of post-thrombolysis hemorrhage.

Previous studies demonstrated that acute ischemic stroke patients had significantly higher plasma median D-dimer levels as compared to healthy controls<sup>[38]</sup>. D-dimer levels increased with increasing severity of stroke and infarct volume and the positive trends existed even after correcting for possible confounding factors<sup>[38]</sup>. Thus, D-dimer concentrations may be considered a direct consequence of marked cerebral infarc-

tion. Although D-dimer levels are significantly associated with cardioembolic stroke, the significance of D-dimer levels in relation to the severity and functional outcomes of other stroke subtypes was investigated by Kim *et al.*<sup>[39]</sup>. Patients with higher D-dimer levels had significantly worse initial functional outcomes, and these worse outcomes were maintained throughout the 9-month follow-up period compared with the low D-dimer group. However, regardless of stroke subtype, D-dimer levels did not influence long-term longitudinal temporal changes of functional outcomes over the 9-month follow-up period<sup>[39]</sup>. In our study too, patients with significantly higher D-dimer levels at admission, had an unfavorable outcome.

In 2005, Anand and Stead<sup>[40]</sup> described that serum NSE level was significantly higher in stroke patients than in controls, and correlated with infarct tissue volume, but did not correlate with the functional outcome. This was explained by the disparity in sampling time because the NSE level has been shown to peak after 4-8 h and hence, a better correlation was expected from delayed sampling. However, in 2013, Zaheer *et al.*<sup>[41]</sup> found that NSE level in day 1 positively correlated with infarct volume and functional neurological outcome at day 30 and negatively correlated with Glasgow Coma Score at presentation. In our study, the mean (SD) time of presentation of the patients who underwent thrombolysis was 3.8 (0.7) h, and they also had elevated serum NSE levels. This signifies the requirement of more multicentre research with a larger sample size to determine the optimum time needed for NSE level to elevate in serum following an acute ischemic stroke.

### Limitations

Small sample size confounded the results of factors predicting the severity and outcome of stroke and resulted in wider CI. Even important risk factors like hypertension, hypercholesterolemia, smoking, and AF could not be established as significant predictors. The short follow up time of 1 month, hindered the evaluation of the long-term neuro-disabilities. The computed tomography machine at our institution does not have the software to determine the infarct volume and so the correlation of the levels of the biomarkers with the volume of infarcted tissue could not be done.

What this study adds: (1) EF between 15%-35% also independently predicts severity and the outcome of ischemic stroke; (2) MABP and CBG at admission significantly predict severity and outcome of ischemic stroke, and meticulous management of these factors may improve the outcome; (3) serum NSE level can rise earlier than 4 h - this fact needs to be validated by other larger studies; (4) fibrinogen level > 478.5 mg/dL at admission significantly predicts the higher chance of post-thrombolysis haemorrhage; and (5) there is no significant difference in the efficacy of intravenous thrombolysis on stroke in various arterial territories.

### DECLARATIONS

#### Authors' contributions

Provided intellectual inputs: Ghosh KC, Bhattacharya R, Mondal GP

Collected data: Ghosh S, Das S, Mahata M

Prepared the manuscript and acted for correspondence: Das S

#### Availability of data and materials

The data and material could be available to readers upon request.

#### Financial support and sponsorship

None.

#### Conflicts of interest

All authors declare that there are no conflicts of interest.

#### Ethical approval and consent to participate

The study was approved by Institutional Ethicals Committee, Calcutta National Medical College, Kolkata and consent was obtained from the patients.

**Consent for publication**

Not applicable.

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Letter to Editor

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# Azygos anterior cerebral artery aneurysm with subarachnoid hemorrhage

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Azygos anterior cerebral artery (ACA) is type I variation of ACA with a reported incidence of < 1% in population<sup>[1]</sup>. This variation predisposes to the formation of aneurysm especially at the bifurcation zone. The aneurysm develops because of double hemodynamic pressure supplying medial surface of both cerebral hemispheres.

However reported incidence of saccular aneurysm in azygos ACA is between 13% and 71%<sup>[2,3]</sup>. It is often associated with other central nervous system (CNS) malformations like agenesis of corpus callosum, hydranencephaly and other vascular malformations<sup>[4]</sup>.

In this paper we report a patient who presented with subarachnoid hemorrhage. Later on we did three dimensional (3D) computed tomography (CT) image. She was then diagnosed as a case of azygos ACA aneurysm.

A 50-year-old female presented with a history of sudden onset of severe headache followed by transient loss of consciousness. There was no previous history of hypertension and diabetes mellitus. During admission she had mild dull aching headache and neck rigidity. There was no focal neurological deficit (Hunt and Hess grade-I).

Four hours after the incident, CT scan of brain revealed subarachnoid hemorrhage in the basal cistern and hematoma in the interhemispheric fissure [Figure 1] which was Fisher CT scan grade 3. After 7 days of the incident repeat CT scan of brain was done which showed diminution of the size of hematoma [Figure 2]. At



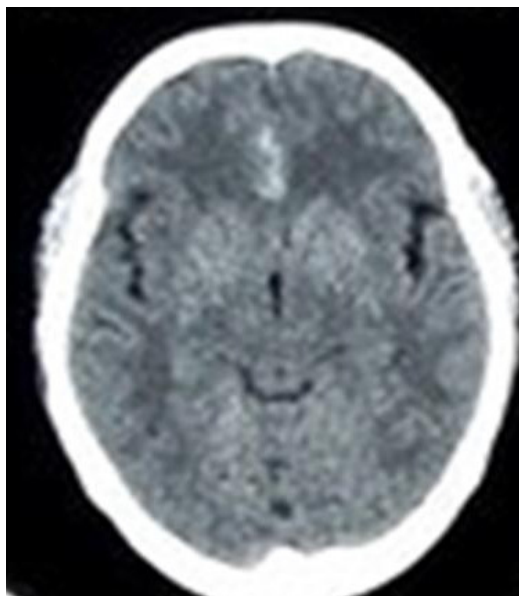
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**Figure 1.** CT scan of brain shows subarachnoid hemorrhage and hematoma in the interhemispheric fissure. CT: computed tomography

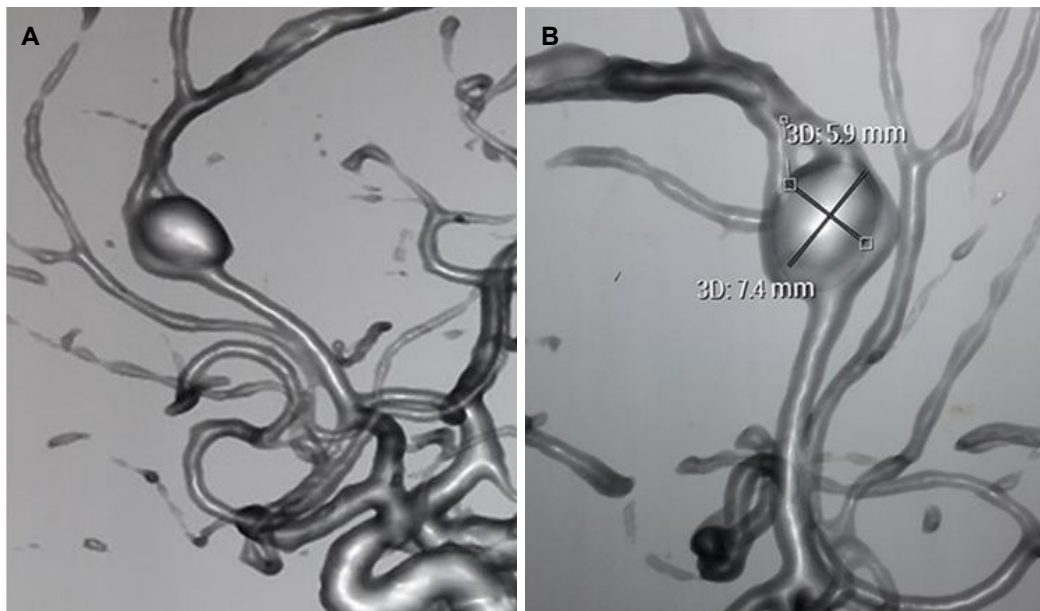


**Figure 2.** After 7 days, CT scan of brain shows diminution of the size of hematoma. CT: computed tomography

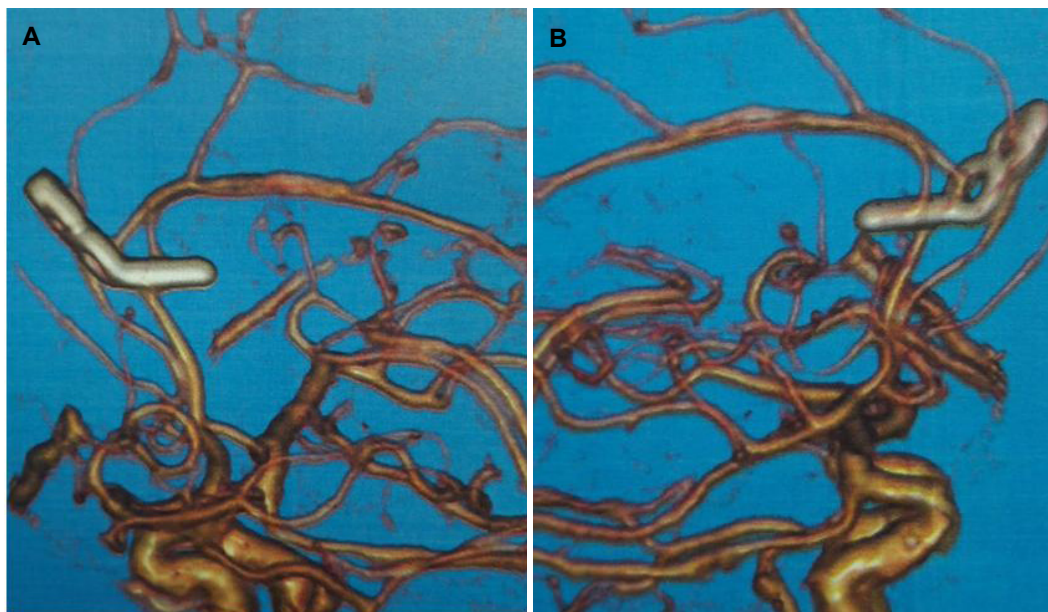
that time, a 3D CT angiogram revealed single saccular aneurysm from the bifurcation zone of azygos ACA [Figure 3A and B].

The patient underwent right paramedian frontal craniotomy and clipping of the aneurysm through anterior interhemispheric approach. After application of temporary clip on unpaired A2 segment, dissection of aneurysm neck was done and a curved fenestrated titanium clip was applied keeping the callosomarginal artery within the fenestration. There was no perioperative rupture of the aneurysm during dissection of aneurysm neck and fundus. Post-operative periods were uneventful.

Two months after the surgery, we did 3D CT angiogram which showed the patency of the vessels and the



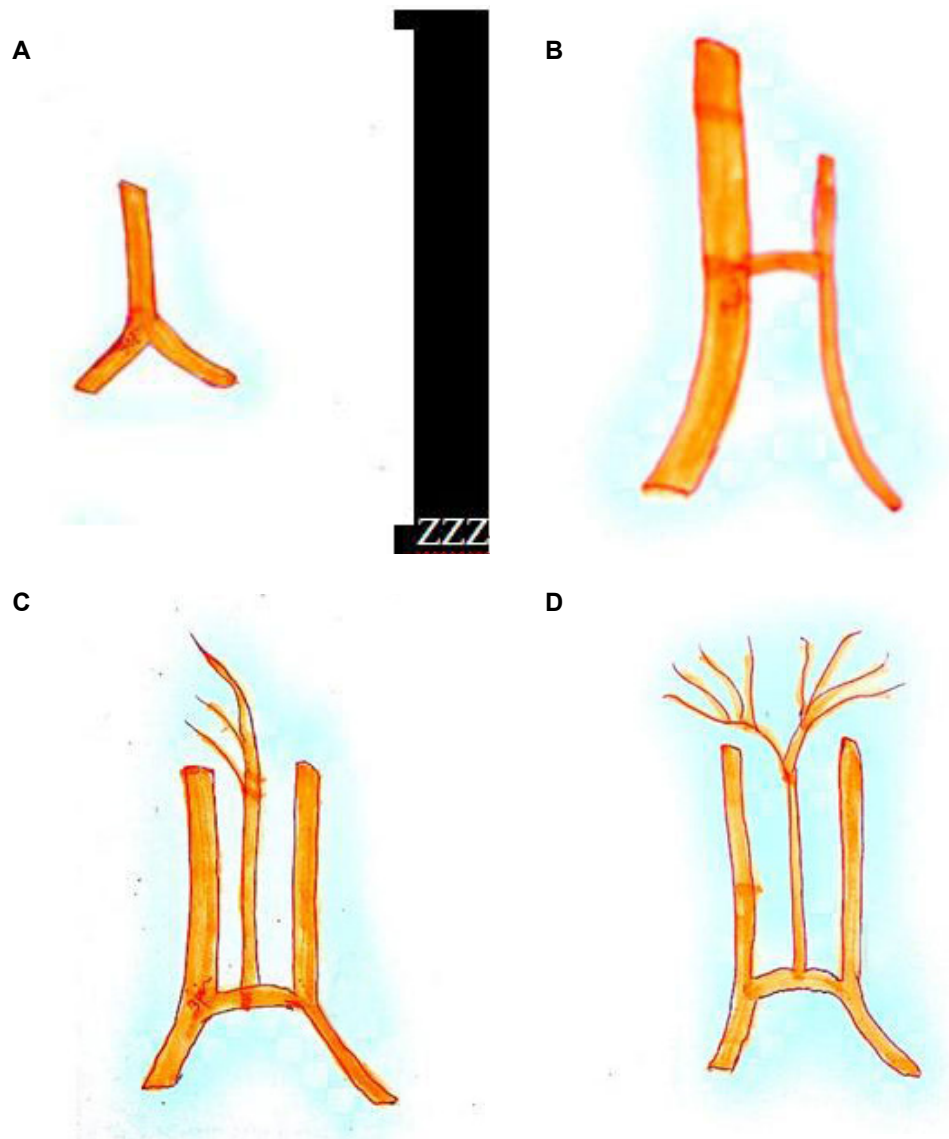
**Figure 3.** (A) 3D CT angiogram demonstrating a saccular aneurysm arising from the bifurcation zone of azygos ACA; (B) 3D CT angiogram showing a 5.9 by 7.4 mm saccular aneurysm. 3D: three dimensional; CT: computed tomography



**Figure 4.** (A and B) Post-operative 3D CT angiogram demonstrating complete obliteration of the aneurysm. 3D: three dimensional; CT: computed tomography

complete obliteration of the aneurysm [Figure 4A and B].

According to Baptista<sup>[7]</sup>, there are three variations of ACA [Figure 5]. Type 1 anomaly [Figure 5A] denotes azygos ACA from which all major vessels arise and supply both hemispheres. In type II variation [Figure 5B] both right and left A2 present and major branches supplying both hemispheres arises from dominant A2. Type III anomaly [Figure 5C and D] denotes accessory ACA arising from anterior communicating artery. In our case, the surgery was done on the basis of 3D CT angiogram findings and azygos nature of ACA was confirmed preoperatively.



**Figure 5.** (A) Azygos ACA in which single ACA feeds into medial surface of both cerebral hemisphere. ACA: anterior cerebral artery; (B) Bihemispheric ACA, in which two ACA, one is dominant with branches extending into contralateral hemisphere; (C and D) Accessory ACA in which a median artery supply either one or both hemispheres. ACA: anterior cerebral artery

Though reported incidence of azygos ACA is  $< 1\%$ <sup>[1]</sup>, Ghanta *et al.*<sup>[6]</sup> reported that 25% cases of distal anterior cerebral artery (DACA) aneurysms were associated with azygos ACA. Katz *et al.*<sup>[7]</sup> found 17% ACA aneurysm among 36 DACA aneurysm cases. The nature of the aneurysm was mostly saccular. Although saccular bilobed aneurysm of ACA was also reported by Jagetia *et al.*<sup>[8]</sup>.

Both surgical clipping and endovascular coiling can be done to manage azygos ACA aneurysm. In our case, temporary clip was applied for 5 min in proximal unpaired A2 segment. Then a dissection of the aneurysm neck was done and finally a permanent clip was applied. There was no neurological deficit in her post-operative period.

In conclusion, we can say that azygos aneurysm can be clipped well without any postoperative morbidity and mortality. Though we tried to manage this case in an emergency basis, we failed due to the patient's poor financial condition.

## DECLARATIONS

### Authors' contributions

Design: Chowdhury D

Literature research, manuscript editing: Chaurasia B

Data analysis, manuscript writing: Ahmed N

Manuscript revision: Chowdhury D, Barua KK

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Presynaptic mechanisms at prefrontal synapses involved in persistent pain

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## ABSTRACT

The cumulative evidence from animal and human studies revealed that the anterior cingulate cortex (ACC) plays essential roles in pain sensation and persistent pain. It has been evident in the ACC synapses of animals that changes in both the presynaptic and postsynaptic function are caused by peripheral nerve injury. Thus far, postsynaptic changes in the ACC following nerve injury have been primarily studied to understand the mechanisms of chronic pain. In recent years, studies focusing on the presynaptic mechanisms in chronic pain have been progressively increased. In this review, I will discuss molecular mechanisms associated with chronic pain and presynaptic form of long-term potentiation. I will also discuss evidence for presynaptic changes in the ACC caused by disease-related pain.

**Keywords:** Presynaptic mechanism, chronic pain, anterior cingulate cortex

## INTRODUCTION

The anterior cingulate cortex (ACC) plays critical roles in the processing of pain information in patients and is involved in behavioral responses to tissue damages that cause pain in animals<sup>[1-8]</sup>. The ACC also contributes to various cognitive processes including decision-making, attention and working memory<sup>[9,10]</sup>. Humans and animals with persistent pain displayed poor performance in decision-making cognitive tasks<sup>[11-13]</sup>. Therefore, the understanding of the cellular and molecular mechanisms of chronic pain is very important in ameliorating the cognitive impairments.



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Sensory inputs from the periphery are conveyed to the dorsal root ganglion and then to the spinal dorsal horn (SDH). The SDH neurons send ascending projection to the thalamus. Subsequently, the outputs from the thalamus synapse on the neurons in the ACC, amygdala and other cortices including the somatosensory and insular cortex<sup>[3,5,14,15]</sup> [Figure 1].

With regard to the synaptic mechanisms of chronic inflammatory and neuropathic pain, it has been proposed that changes in both the presynaptic and postsynaptic function play essential roles<sup>[8,16]</sup>. To date, a number of studies have shown that tissue injury- or nerve damage-caused central sensitization, a similar phenomenon like long-term potentiation (LTP), in the ACC could contribute to the persistent pain<sup>[7,8]</sup>. Since it has generally been believed that postsynaptic mechanisms are crucial for the LTP expression, postsynaptic changes following nerve injury in the ACC synapses have been primarily studied to understanding the mechanisms of chronic pain. In behavioral experiments using genetic and pharmacological approaches, inhibiting postsynaptic  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated synaptic plasticity is shown to be sufficient to produce analgesic effects<sup>[17]</sup>. These data indicate that postsynaptic LTP in the ACC is involved in chronic pain. Compared to studies focusing on postsynaptic mechanisms, those focusing on presynaptic mechanisms which contribute to persistent pain are relatively few. However, presynaptic changes in the ACC underlying chronic pain have been progressively elucidated in recent years. In this review, I will discuss studies regarding the molecular mechanisms that play pivotal roles in chronic pain and presynaptic form of long-term potentiation (pre-LTP) in the ACC. In addition, I discuss presynaptic changes associated with disease-related pain in the ACC.

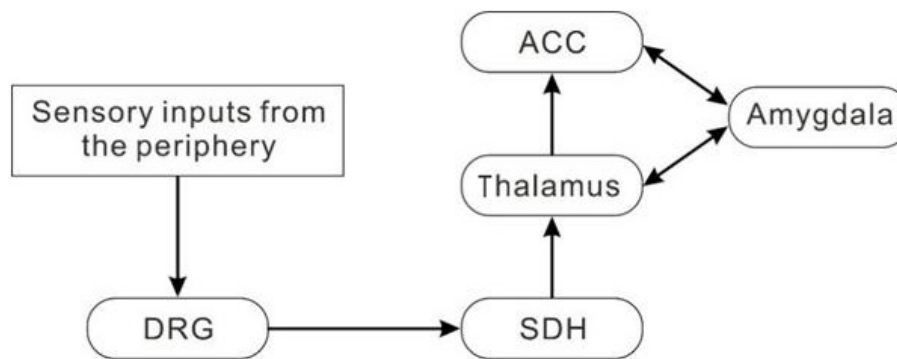
### **Molecular mechanisms of presynaptic changes in the ACC following nerve injuries and inflammation**

#### *Calcium/calmodulin-stimulated adenylyl cyclase*

Cyclic adenosine monophosphate (cAMP) is a nucleotide that acts as a key second messenger in a number of physiological functions including chronic pain, learning and memory, emotional fear and drug abuse<sup>[18,19]</sup>. The adenylyl cyclase (AC) is the important enzyme that converts ATP to cAMP. The AC family is composed of nine membrane-bound isoforms (AC1-9) and one soluble isoform (sAC). These isoforms are differentially distributed in the body, and each AC isoform has distinct physiological functions<sup>[20]</sup>. Among AC1-9 and sAC, AC1 and AC8 are the key AC isoforms that respond positively to calcium-calmodulin<sup>[20]</sup>. AC1 is 4 to 5 times more sensitive to an increase in calcium concentration than AC8. It has been shown that AC1 is abundantly expressed in the mouse ACC neurons<sup>[21]</sup>. We have previously demonstrated that deletion of AC1 and AC8 genes significantly reduced pain sensitization in mice with chronic inflammatory pain<sup>[22]</sup> and in those with chronic neuropathic pain<sup>[16,21]</sup>. In ACC synapses of mice with inflammatory pain, the enhancement of presynaptic transmitter release was suppressed by inhibition of AC1 and/or AC8<sup>[22]</sup>. Thus, presynaptic AC1 and/or AC8 could be the key molecules that contribute to the enhancement of both the probability of transmitter release and the number of available vesicles in response to inflammatory pain. Subsequently, AC1 and/or AC8 would activate protein kinase A (PKA) and then phosphorylate cAMP response element binding protein<sup>[21]</sup>. In addition, we have shown that AC1 plays essential roles in both the presynaptic and postsynaptic changes in ACC synapses in the mouse spinal nerve ligation model of neuropathic pain<sup>[16]</sup>.

An alysia octopamine receptor (Ap oa1) is G protein-coupled and selectively activates cAMP/PKA pathway<sup>[23]</sup>. By using transgenic mice heterologously expressing Ap oa1, we have previously examined whether and how cAMP in the ACC synapses is involved in the presynaptic modulation of neurotransmitter release. We found that the activation of Ap oa1 by octopamine augmented glutamatergic synaptic transmission in the ACC synapses<sup>[24]</sup>. Also, behavioral responses to inflammatory pain were apparently facilitated by bilateral microinjection of octopamine into the ACC<sup>[24]</sup>. These findings provide the evidence that the presynaptic modulation by cAMP contributes to chronic pain caused by peripheral inflammation. Therefore, AC1 may be potential therapeutic targets for treatment of chronic pain. Indeed, intraperitoneal or





**Figure 1.** A possible neuronal network for the anterior cingulate cortex (ACC) in pain transmission. Sensory inputs from the periphery are conveyed to the dorsal root ganglion (DRG) and then to the spinal dorsal horn (SDH). Some of the SDH neurons send ascending projection to the neurons in the thalamus. Subsequently, the outputs from the thalamus synapse on the neurons in the amygdala, ACC and other cortical neurons (not shown)

oral application of NB001, an AC1 inhibitor, reduced pain sensitivity in neuropathic pain mice without no apparent side effects<sup>[25,26]</sup>.

#### *Inflammation-related factors*

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a potent prototypic inflammatory cytokine and is produced by a wide variety of cells including neuronal and glial cells<sup>[27,28]</sup>. TNF- $\alpha$  increases glutamatergic synaptic transmission by inhibiting glutamate transporters expressed in glial cells and/or by increasing the surface expression of AMPA and N-methyl-d-aspartate (NMDA) receptors<sup>[29]</sup>. In addition, TNF- $\alpha$  is known to serve as a trigger for other cytokines in response to inflammation or injury. There is a large number of evidence that TNF- $\alpha$  is responsible for the development and maintenance of chronic inflammatory and neuropathic pain<sup>[30]</sup>. A previous finding proposed that TNF- $\alpha$  may induce acute mechanical sensitization of peripheral nociceptors by acting on TNF receptor 1<sup>[31]</sup>. In the mouse ACC, the protein level of TNF- $\alpha$  was significantly increased following hind-paw administration of complete Freund's adjuvant<sup>[32]</sup>. Together with this finding, the glutamatergic synaptic transmission was significantly enhanced by TNF- $\alpha$  in the ACC synapses. These data suggest that that presynaptic changes caused by peripheral nerve inflammation are partly brought about by the increased TNF- $\alpha$  in the ACC synapses.

Chemokines that belong to proinflammatory cytokines play essential roles in cell growth, immune system, cell development and inflammation. The two chemokine superfamilies, the CC and the CXC chemokines, are thought to be important for these functions<sup>[33]</sup>. Five receptors for CXC chemokines and eight receptors for CC chemokines have been characterized<sup>[34]</sup>. Among CXC chemokines, interleukin-8 (IL-8), known as CXCL8, is a critical proinflammatory CXC chemokine that is associated with the neutrophil recruitment and degranulation in inflammatory response<sup>[35]</sup>. In response to inflammatory responses within the central nervous system, IL-8 is produced by activated microglia and astrocytes<sup>[36]</sup> and may affect excitability of neurons through modulating intercellular interaction between glial cells and neurons. It has been demonstrated that the expression level of IL-8 was increased in the ACC synapses of mice with persistent inflammatory pain, and the glutamatergic synaptic transmission was enhanced in the ACC synapses<sup>[37]</sup>. These results indicate that up-regulation of IL-8 in the ACC synapses contributes to the enhanced excitatory synaptic transmission in mice with chronic inflammatory pain.

#### *Synaptic proteins*

Neurotransmitters are released from presynaptic nerve terminals by exocytosis of the synaptic vesicles. During this event, many synaptic proteins interact with cytosol and plasma membrane proteins<sup>[38]</sup>. Cumulative evidence has shown that synaptic proteins such as synaptobrevin, synaptogyrin, synaptophysin,

synaptotagmin, syntaxin and synaptosome-associated protein-25 (SNAP-25) are involved in the synaptic plasticity in the central nervous system<sup>[39-41]</sup>. Increases in synaptic vesicle proteins including synapsin, synaptophysin and synaptotagmin contribute to the enhanced synaptic potentiation in the hippocampal neurons<sup>[42,43]</sup>. Furthermore, in the spinal cord of chronic neuropathic pain rat models, the number of presynaptic boutons that are synaptophysin-immunoreactive was significantly increased<sup>[44]</sup>. In synaptosomal fractions obtained from the medial prefrontal cortex of neuropathic pain rat models, expression levels of presynaptic proteins (including synaptotagmin, synaptophysin, syntaxin, synaptobrevin and SNAP-25) were significantly enhanced, compared to those obtained from sham rats<sup>[45]</sup>. In addition, the electron microscopy revealed that synaptic vesicles in the synaptosomes were significantly increased in the medial prefrontal cortex of neuropathic pain rat models<sup>[45]</sup>. These observations suggest that the enhancement of both the number and availability of synaptic vesicles play critical roles in the enhancement of excitatory synaptic transmission in the medial prefrontal cortex of neuropathic pain rat models.

SOS3-interacting protein 3 (SIP3), which is known as a SNAP25-interacting protein 30, was first identified in the hair cells of guinea pig cochlea<sup>[46]</sup>. This novel protein is composed of 266 amino acids is one of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors and plays essential roles for regulated exocytosis of synaptic vesicles in neurotransmission<sup>[47]</sup>. SIP30 is highly expressed in various brain regions including the cortex<sup>[46]</sup>. SIP30 was previously shown to be associated with the modulation of pain. For example, the increase in the expression of SIP30 in the spinal cord of chronic constriction injury rats was involved in the formation and maintenance of neuropathic pain<sup>[48]</sup>. In addition, the same group revealed that chronic constriction injury induced an increase of SIP30 in both sides of the ACC together with the behavioral hypersensitivity<sup>[49]</sup>. Interestingly, they showed that knockdown of SIP30 by lentiviral vector-mediated short hairpin RNA in the ACC significantly suppressed not only the behavioral hypersensitivity but also the glutamate release within the ACC. These results suggest that SIP30 is critical for the increased excitatory synaptic transmission in the ACC.

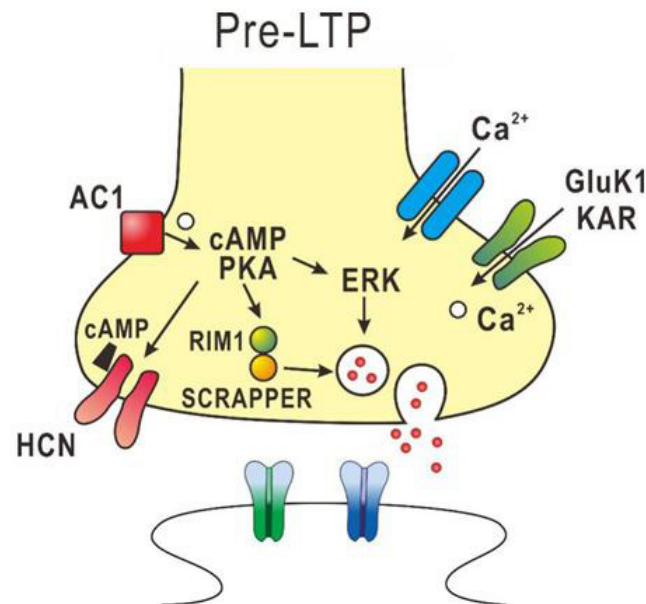
### **Presynaptic changes in ACC synapses of animals with disease-related pain**

#### *Diabetes mellitus*

Diabetes mellitus is known as one of metabolic diseases and is caused by deficiency or diminished effectiveness of endogenous insulin. The high blood sugar levels resulting from the reduced insulin produces several symptoms such as increased hunger, frequent urination and increased thirst. If left untreated, diabetes mellitus causes many acute and chronic complications<sup>[50]</sup>. Peripheral neuropathy is the most common and debilitating complication of diabetic patients<sup>[51]</sup>. Thus, a number of studies revealed that diabetes-related plasticity occurs in the spinal nociceptive neurons<sup>[52-54]</sup>. In addition to the spinal cord, diabetes mellitus causes plasticity in the supraspinal brain regions pertinent to the processing of pain information<sup>[55,56]</sup>. In the ACC of the streptozotocin-induced diabetic rats, there was a significant reduction in paired-pulse facilitation compared to the control mice<sup>[56]</sup>, suggesting that an enhanced presynaptic glutamatergic synaptic transmission occurs in the ACC neurons of the diabetic mice. Therefore, it is strongly suggested that presynaptic changes in the ACC are critically involved in the diabetes-induced pain.

#### *Hypothyroidism*

Two thyroid hormones, triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>), are primarily responsible for regulation of metabolism and play essential roles in the development and activity of the central nervous system<sup>[57]</sup>. The thyroid status of neonates and children has a significant long-term impact on locomotor activity and cognition<sup>[57]</sup>. Indeed, congenital hypothyroidism, which is characterized by deficiencies in thyroid hormone production in newborn infants, causes mental retardation in children<sup>[58]</sup>. In addition, adult-onset hypothyroidism which is caused by insufficiency of thyroid hormones in the adulthood decreases cognitive functions<sup>[59]</sup>. The interaction between pain and thyroid hormones has been progressively elucidated<sup>[60]</sup>. In athyreotic patients, the pain sensitivity was associated with the thyroid status<sup>[61]</sup>. It has also been reported



**Figure 2.** Diagram of the potential mechanisms in presynaptic form of long-term potentiation (pre-LTP) in the anterior cingulate cortex (ACC). The presynaptic  $Ca^{2+}$  influx via GluK1 KAR s leads to activation of the adenylyl cyclase 1 (AC1)-PKA pathway. Then cAMP binds to the HCN channel to increase its sensitivity and activates PKA to enhance the release of glutamate, presumably through activation of extracellular signal-regulated kinase (ERK). The enhanced PKA activity is likely to activate SCRAPER and RIM1 to regulate vesicle recycling, which is required for pre-LTP in the ACC synapses. KAR: kainate receptor; PKA: protein kinase A; cAMP: cyclic adenosine monophosphate; HCN: hyperpolarization-activated cyclic nucleotide-gated

that an adequate supply of thyroid hormones from mother rats is necessary to acquire a normal nociceptive function in the offspring into adulthood<sup>[62]</sup>. Using experimental hypothyroidism mice that were treated with potassium perchlorate and methimazole in the drinking water, it was investigated whether and how synaptic transmission and pain perception in the ACC are modulated by thyroid hormones<sup>[63]</sup>. In hypothyroid mice, the thermal pain thresholds were decreased. Furthermore, whole-cell patch-clamp recordings showed that the frequency of miniature excitatory postsynaptic currents was increased while that of miniature inhibitory postsynaptic currents was decreased in ACC neurons. Treatment with thyroid hormones (T3 or T4) markedly reduced hypersensitivity to noxious stimuli and reversed the synaptic alterations. Therefore, it is likely that the hypersensitivity to noxious heat observed in the hypothyroid mice is caused by presynaptic changes in the ACC synapses.

### Pre-LTP in ACC synapses and its molecular mechanisms

In ACC synapses of adult mice, pre-LTP can be induced by low-frequency stimulation and is proposed to be involved in pain-triggered anxiety<sup>[64,65]</sup>. The pre-LTP in the ACC synapses is reduced in chronic inflammatory and nerve injury model mice<sup>[64]</sup>. The induction of pre-LTP in the ACC is dependent on GluK1 containing kainate receptors, calcium-stimulated AC1 and extracellular signal-regulated kinase (ERK), but is independent of NMDA receptors, metabotropic glutamate receptors and protein kinase M zeta<sup>[64-66]</sup>. Activation of AC1 increases cAMP, which binds to hyperpolarization-activated cyclic nucleotide-gated channels to enhance its sensitivity. Also, AC1 activates PKA to enhance the release of glutamate, presumably through activation of ERK. Recently, it was found that SCRAPER which is an E3 ubiquitin ligase expressed in presynaptic terminals is required for ACC pre-LTP<sup>[67]</sup>. The pre-LTP in the ACC synapses may be necessary for the activation of both presynaptic molecules (e.g., RIM1) and the ubiquitin-proteasome system including SCRAPER protein<sup>[67]</sup>. Provided that cortical pre-LTP is involved in pain-triggered anxiety and fear, these molecules are potential molecular targets for relief from pain-triggered anxiety and fear. The molecular mechanisms involved in pre-LTP are summarized in Figure 2.

## CONCLUSION

Peripheral nerve injuries and peripheral inflammation can cause extensive changes in pain processing in the central nervous system. Many *in vitro* and *in vivo* studies have proved compelling evidence that changes in both presynaptic and postsynaptic function are responsible for chronic pain. It is believed that the key presynaptic and postsynaptic events in chronic pain in the central nervous system are synaptic plasticity triggered by peripheral nerve injury and ongoing unusual sensory inputs afterwards. Therefore, understanding of molecular and cellular mechanisms underlying the pain-induced synaptic plasticity will provide new therapeutic targets for treating persistent pain in patients. At present, little is understood about molecular mechanisms underlying presynaptic changes in chronic pain state, compared to those underlying postsynaptic changes. Thus, it is necessary to further elucidate the cellular and molecular mechanisms underlying presynaptic changes in the ACC of chronic pain mice. Treatment of chronic pain is difficult because current available drugs for chronic pain induce unwanted side effects or supply insufficient analgesia. Thus, studies focusing on the molecular mechanisms of presynaptic changes in the ACC of chronic pain mice could lead to the development of effective novel analgesics on treating chronic pain. Although it has been reported that pre-LTP in the ACC synapses is involved in pain-triggered anxiety and fear<sup>[64,65]</sup>, the molecular and cellular mechanisms for the pre-LTP are not well understood. Understanding of these mechanisms for pre-LTP in the ACC synapses will help to develop new therapeutic strategies for pain-triggered anxiety and fear.

## DECLARATIONS

### Authors' contributions

The author contributed solely to the article.

### Availability of data and materials

Not applicable.

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### Conflicts of interest

The author declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Original Article

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# Changes in microglia activity of rat brain induced by *Macrovipera lebetina obtusa* venom

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## ABSTRACT

**Aims:** The microglia activity of rat brain following exposure of the *Macrovipera lebetina obtusa* venom was investigated.

**Methods:** Histochemical analysis of brain microcirculatory bed staining by  $\text{Ca}^{2+}$  ATPase method for variable doses after intraperitoneal injections given for different time periods was used. The hemorrhagic activity of snake venom metalloproteinases was tested. Toxicological data were calculated using Behrens and Miller-Tainter methods. Surface, size of brain microglial cells (MGCs) and staining intensity were quantified using ImageJ software.

**Results:** The vasodestructive action of the venom resulted in changes in ATPase activity. The intensity of staining of rat brain microcirculatory bed was venom dose-, and time-dependent. Increased activity of MGCs in hemorrhagic loci of different regions of venom affected brain was also demonstrated.

**Conclusion:** The activation of microglia and changes of its form, size, and position strongly correlates with hemorrhage-induced cerebrovascular damage.

**Keywords:** *Macrovipera lebetina obtusa* venom, toxicity  $\text{LD}_{50}$ , hemorrhage, metalloproteinase, rat brain microcirculatory bed, microglia



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## INTRODUCTION

The Vipers are the lone family of venomous snakes found in Armenia. The main action of viper venoms is related to protein-degrading proteases, which cause local necrosis, systemic cardiovascular damage, and disruption of the blood-clotting system<sup>[1]</sup>. The whole venom of Caucasian Levantine viper [syn.: blunt-nosed viper, *Macrovipera lebetina obtusa* (MLO)] contains many enzymatically active proteins and different polypeptides [Figure 1] with a variety of effects on cells and organs, including pronounced hemorrhagic effect<sup>[2,3]</sup>. *Macrovipera lebetina* species venom contains snake venom metalloproteinases (SVMP)<sup>[4,5]</sup> which are Zn<sup>2+</sup>-containing, Ca<sup>2+</sup>-dependent enzymes that differ in domain structure: some of them contain just metalloproteinase domain, others contain metalloproteinase domain and non-catalytic domains. MLO venom contains only two out of four types of SVMPs: P-I and P-III<sup>[2]</sup>. Metalloproteinase P-I has only catalytic domain<sup>[6,7]</sup> and constitutes more than one-quarter of the whole MLO venom.

The P-III is characterized by proteolytic enzyme property, high ability to bind to the substrate<sup>[6,8]</sup> and high hemorrhagic activity (with rare exceptions<sup>[9]</sup>). Together PI and PIII are making up approximately one-third of the entire contents of the MLO venom. SVMPs, which are secretory proteinases, cause hemorrhagic injuries, splitting the components of the basement membrane and disrupting their interaction with endothelial cells<sup>[10]</sup>. In addition, they induce muscle necrosis, skin damage, and inflammation. Some SVMPs induce apoptosis in human umbilical vein endothelial cells<sup>[11]</sup>. Moreover, SVMPs interact with matrix metalloproteinases (MMPs) of a prey. MMPs are carrying out tissue remodeling, angiogenesis, cell proliferation, migration, differentiation, apoptosis, and in curbing the growth of tumors<sup>[12]</sup>. They are involved in the cleavage of membrane receptors, ligand release, as well as in the activation/deactivation of the chemokines and cytokines<sup>[13]</sup>. SVMP P-I and P-III can trigger secretion of prey's MMP-2 and MMP-9, which together with venom MPs may destroy extracellular matrix or VE-cadherins<sup>[4]</sup>. These and other cadherins ( $\beta$  and  $\gamma$ ) are denatured leading to increased intracellular permeability<sup>[11]</sup>. During such damage of brain tissue, microglia plays a critical role in the protection of brain from the action of harmful factors of the venom<sup>[14-16]</sup>.

Mechanisms by which different doses of snake venom, and particularly, SVMPs affect or disrupt the microvasculature of brain and cause hemorrhage have not been fully elucidated. The goal of our investigation was to assess hemorrhagic effects of MLO venom on rat brain given at different doses and for different periods when administered via intraperitoneal (IP) route.

## METHODS

### Animals

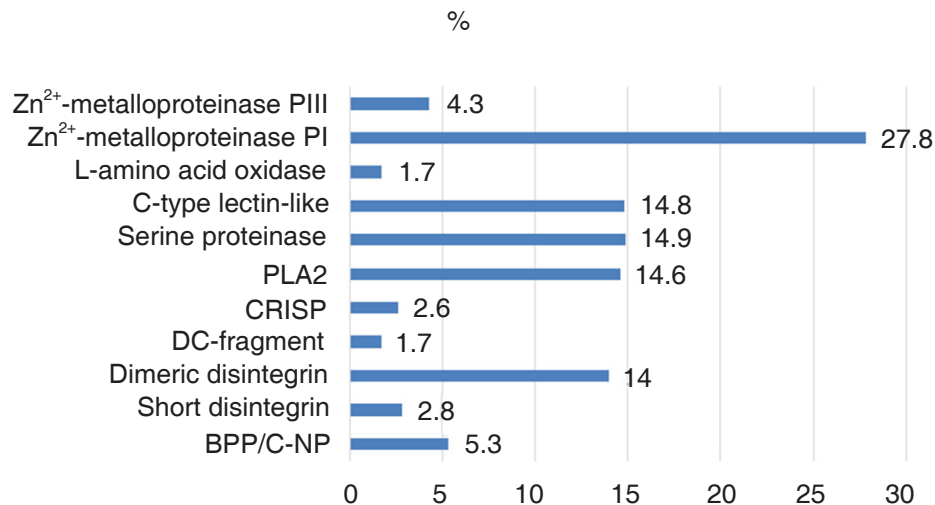
This study was conducted in accordance with “Principles of Laboratory Animal Care” and as carried out in accordance with the European Communities Council Directive of September 22 2010 (2010/63/EU). Sixty-two adult Wistar albino male rats were used in this study.

### Reagents

MLO venom was milked and dried in the Laboratory of Physiologically Active Substances Purification, Certification and Standardization (PAS PCS) of Orbeli Institute of Physiology of National Academy of Sciences of Armenia. Snakes were supplied by the licensed catcher “GEBEVSS” Ltd., Armenia. Other reagents were purchased from “Sigma-Aldrich”, “Merck” “Reanal” and “H. Lundbeck A/S” companies as indicated. All other chemicals were of analytical or sequencing grade.

### Verification of venom hemorrhagic activity *in vivo*

The hemorrhagic activity of SVMPs was tested *in vivo* by a modified method of Kondo *et al.*<sup>[17]</sup>. In brief, 2 rats (around 250 g) were anesthetized with Nembutal (pentobarbital, at a dose of 40 mg/kg) and 4 cm<sup>2</sup> of dorsal skin was chemically depilated (“Veet”, Reckitt and Benckizer, France). 0.1 mL aliquots of a



**Figure 1.** Percentile of venom of *Macrovipera lebetina obtusa* components<sup>[21]</sup>. (BPP/C-NP: bradykinin potentiating/C-natriuretic peptide; CRISP: cysteine-rich secretory protein; PLA2: phospholipase A<sub>2</sub>)

physiological solution containing 2.0 mg venom were injected subcutaneously (SC). After 2 h the animals were euthanized and dorsal skin was removed. Hemorrhagic spots were identified and counted on the inner surface of the skin.

#### Verification of venom toxicity *in vivo*

Experiments were done on 30 adult male rats weighing 220-250 g to determine MLO venom toxicity LD<sub>50</sub> (IP injection). Five doses were chosen for determination of LD<sub>50</sub> starting from 100% vitality to 100% mortality. In our study for estimation of LD<sub>50</sub> of venom, 5 doses were given intraperitoneally to 5 groups of rats, 6 in each group. Each rat in the first group was injected IP with MLO venom at a dose of 1 mg/kg. In the second group rats were injected with 2 mg/kg, the third: 3 mg/kg, fourth: 4 mg/kg and fifth: 5 mg/kg respectively. The number of dead animals was recorded at 24 h and the percent of mortality in each group was calculated. Percentage mortalities were transformed to probits and corrected formula for 0% mortality [100(0.2/N)] and 100% mortality [100(N-0.25/N)] was used. Approximate standard error was calculated from following formula:

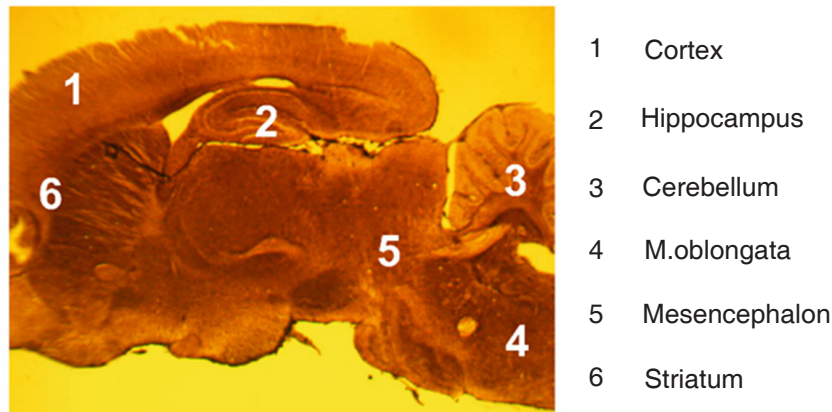
$$\text{Approx. SE of LD}_{50} = (\text{LogLD}_{84} - \text{LogLD}_{16}) / (2N)^{1/2},$$

where N is number of animals in each group.

LD<sub>16</sub>, LD<sub>84</sub> and final data were calculated using table of probits for Behrens and Miller-Tainter methods<sup>[18]</sup>.

#### Histochemical studies

Thirty rats were used for histochemical studies. The animals were kept in the nursery for laboratory animals of Institute of Physiology NAS of Armenia. Daylight and diet were conditioned in accordance with the Animal Protocol of the Institute. Venom was injected in at 2.0, 3.0 and 5.0 mg/kg IP, which was approximately equal to 1.0, 1.5 and 2.5 LD<sub>50</sub> for MLO venom. Animals were divided into 5 groups with 6 animals in each: Group #1 was a control group with intact rats that have only been anesthetized before they were sacrificed. The other 4 experimental groups were set as follows: Group #2, #3 and #4 were injected with different doses of MLO venom (1.0 LD<sub>50</sub>, 1.5 LD<sub>50</sub>, and 2.5 LD<sub>50</sub> respectively) followed by extraction of the brain 2 h post-injection. Group #5 was injected with a dose of MLO venom (2.5 LD<sub>50</sub>), however, brains were isolated 1 h post-injection.



**Figure 2.** Investigated areas of rat brain

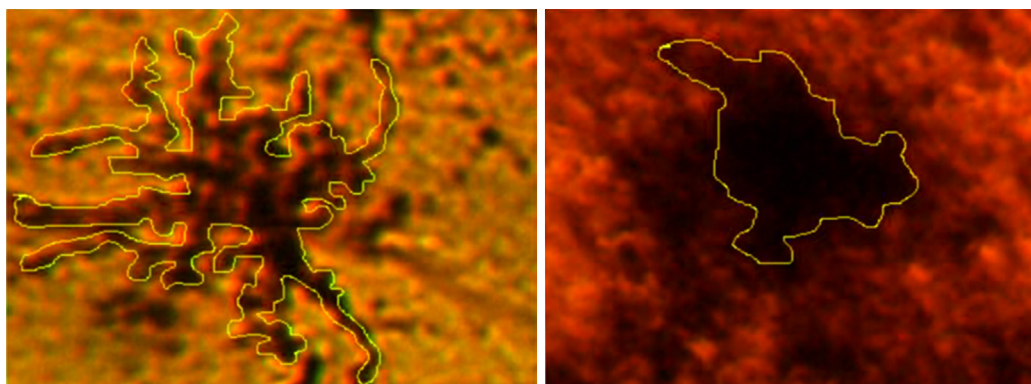
The animals were anesthetized (pentobarbital, at a dose of 40 mg/kg) and sacrificed by decapitation after one or two hours. The brains were excised, fixed in 5% solution of formaldehyde, frozen, and then sliced. The method developed by Chilingaryan *et al.*<sup>[19]</sup> was used. This method is very practical for identifying intra-organ microcirculatory bed. Vessel staining is based on the activity of phosphatases (specifically ATPases) and precipitation of released phosphate with calcium ions at high pH (pH 10.5-11.5). The resulting precipitate is subsequently converted to brown lead sulfide. The method provides selective, clear, and contrast detection of vascular capillary network in thick (60-100  $\mu$ m) sections of the brain. A major advantage is the possibility of simultaneous identification of various branches of the microcirculatory bed (arterioles, capillaries, venules). The intact brain tissue vessels were mostly semitransparent, so an increase of vessels staining density was estimated an indicator of venom action.

A modification of the original Chilingaryan *et al.*'s<sup>[19]</sup> method is described in details below.

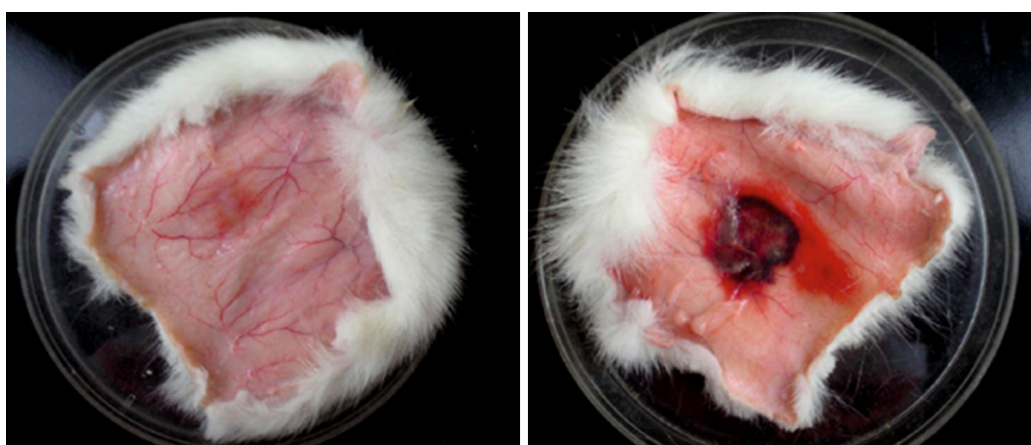
The brains of rats were fixed in a 5% solution of neutral formalin (using tap water) for 24 h. (Slices of 90  $\mu$ m were prepared using freeze microtome). Slices were collected in a saline solution and transferred into incubation buffer [4 mL 4 N 25% ammonia solution; 2 mL 0.1 mol/L calcium chloride solution; 2 mL adenosine triphosphate (ATP) solution (0.1 mol/L fresh prepared solution of disodium salt of ATP) + 12 mL distilled H<sub>2</sub>O] for 1.5 h, then washed twice in distilled water for 2-5 min/wash and then immersed into a replacement lead solution, prepared as follows: to 100 mL of water add 2 drops of glacial acetic acid, then 2 g lead acetate, and then add 10 mL 1 mol/L acetate buffer (pH 6.2) and 15 mL 8% solution of ammonia acetate). The mixture is suitable for a long time and can be used repeatedly. In a specified mixture, slices remain for 1.5 h. After washing in distilled water for 2-5 min, slices were immersed into 1% solution of sulfosalicylic acid on 2-3 min depending on the thickness of the slices. Thereafter the slices were washed twice in distilled water for 2-5 min/wash and were immersed into 2%-5% solution of sodium sulfide for 1-2 min. After all slices were rinsed in distilled water and transferred on glass. Then slices were dried for 1.5 h and overlaid with Canada balsam.

Different regions of rat brain were investigated: *medulla oblongata*, *mesencephalon*, *cerebellum*, *striatum*, *hippocampus*, and *cortex* in accordance with rat brain atlas<sup>[20]</sup>. Sagittal sections of rat brain allowed observation of all regions on the same histological slide [Figure 2].

Photomicrographs of brain slices were done using digital eyepiece FMA050 AmScope UCMOS09000KPB and lenses x6, x9, x20 and x40. Surface, size of brain microglial cells (MGCs) and staining intensity were quantified using ImageJ software<sup>[21]</sup> [Figure 3]. Pixels were identified by converting the original color images to an 8-bit grayscale, followed by the application of an automatic threshold function of the ImageJ software.



**Figure 3.** A sample of microglial cell contouring for footprint and linear dimensions measurement. The left slice - contoured cell of the brain, 1 h after injection, the right slice - 2 h after injection



**Figure 4.** No hemorrhage is detected on the skin of sham-injected animals (left), however animals injected with 2.0 mg/0.1 mL of *Macrovipera lebetina obtusa* venom demonstrate significant hemorrhage after 2 h (right)

## Statistics

The area, the perimeter and staining intensity of brains' MGCs were evaluated. Six separate viewfields were assessed for each intact and experimental animal. Means and standards error/deviation were quantified using ImageJ software and calculated for all groups using Student *t*-test. *P*-values less than 0.05 were considered as statistically significant.

## RESULTS

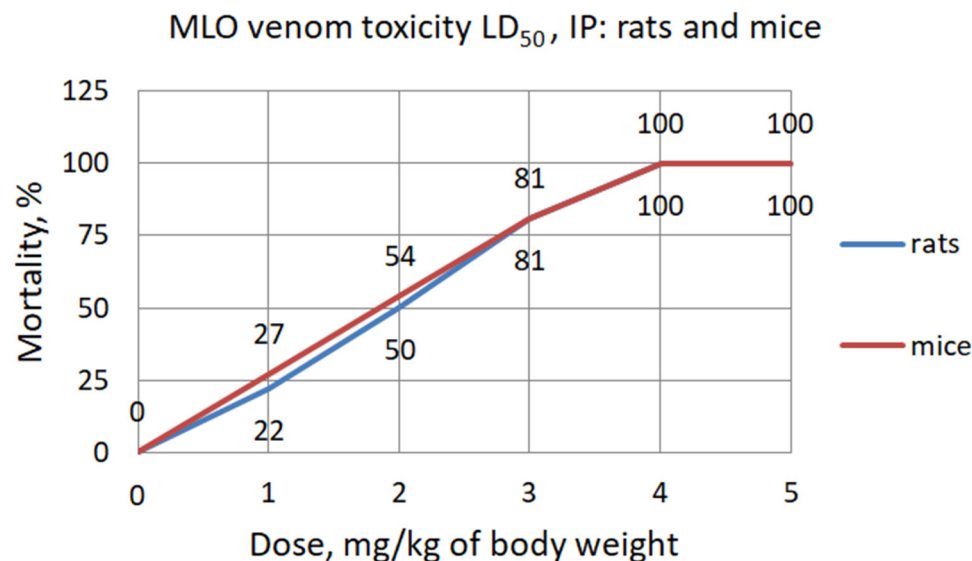
### Hemorrhagic activity investigations

First, we investigate the hemorrhagic activity of MLO venom. To that end, MLO venom was injected SC on the back of rats (2.0 mg/0.1 mL). After 2 h, the back skin was removed and the inner surface of the skin was examined for the presence of hemorrhagic spot [Figure 4].

### Toxicological investigations

The median lethal dose (LD<sub>50</sub>) of MLO venom for intravenous injection (IV) was determined previously by two different groups. Archundia and coworkers in 2011 determine the LD<sub>50</sub> of MLO venom to be 30.1 µg/mouse<sup>[22]</sup>, whereas Kurtović *et al.*<sup>[23]</sup> found LD<sub>50</sub> to be 18.4 µg/mouse. Considering this discrepancy and the absence of data on the IP route of venom administration in rats, we have tested and established toxicity (LD<sub>50</sub>) for IP injection of MLO venom for mice and rats.





**Figure 5.** The toxicity of *Macrovipera lebetina obtusa* venom in mice and rats according to Behrens method

**Table 1.** *Macrovipera lebetina obtusa* venom toxicity LD<sub>50</sub> calculated according to Miller-Tainter method

Calculation for rats	MLO venom	Calculation for mice	MLO venom
Behrens calculated LD <sub>50</sub>	2.0	Behrens calculated LD <sub>50</sub>	1.85
LD <sub>84</sub>	3.15	LD <sub>84</sub>	3.1
LD <sub>16</sub>	0.7	LD <sub>16</sub>	0.6
Standard error (SE)	0.1	SE	0.2
Miller and Tainter calculated LD <sub>50</sub>	1.86	Miller and Tainter calculated LD <sub>50</sub>	1.74
LD <sub>50</sub> ± SE	1.86 ± 0.1	LD <sub>50</sub> ± SE	1.74 ± 0.2

The toxicity of MLO crude venom in mice according to Behrens method was 1.85 mg/kg and calculated by Miller-Tainter method was (LD<sub>50</sub> ± SE) 1.74 ± 0.2 mg/kg. The toxicity of MLO crude venom in rats according to Behrens method was 2.0 mg/kg and by Miller-Tainter method was (LD<sub>50</sub> ± SE) 1.86 ± 0.1 mg/kg [Figure 5 and Table 1].

### Histochemical investigations of changes in microglia activity and microcirculatory bed of rat brain

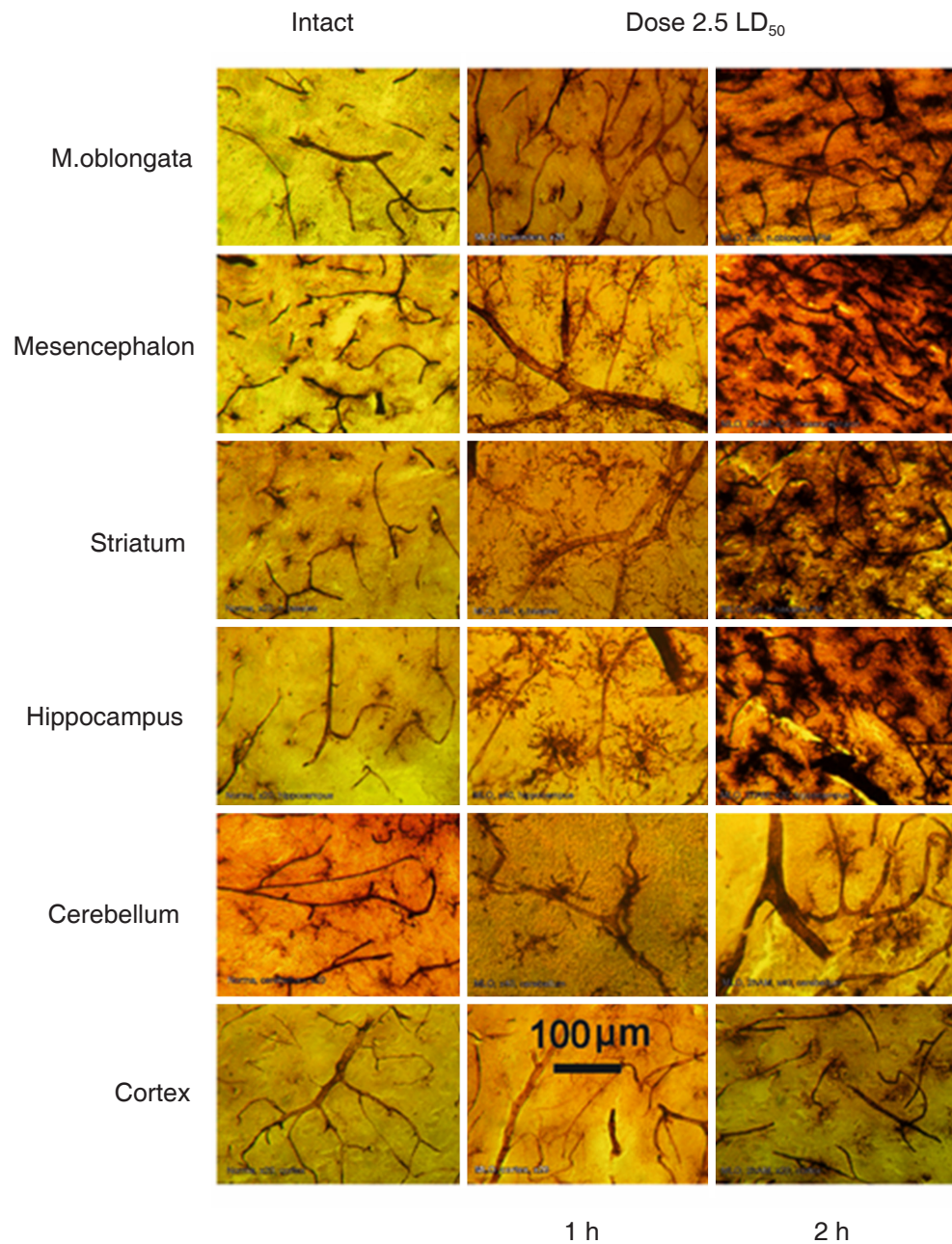
It is well known that main effect of venom depends on enzymatic (both time and dose-dependent) and non-enzymatic activity of specific low molecular weight peptides (dose dependent)<sup>[24]</sup>. Therefore, we performed histochemical investigations aimed at revealing changes in rat brain microcirculatory bed and activity of microglia. These experiments were done using variable doses of MLO venom given for indicated periods.

We compared data obtained after 1 h and 2 h after venom injection. After 2 h the results demonstrated well-defined picture of affected capillaries of the microcirculatory bed of blood vessels in various regions of the brain with activated, migrating MGC as dark amoeboid spots, especially at the points of contacting with vessels. The most venom affected blood vessels were observed in the deep structures of the brain and striatum. Blood vessels in the cerebellar and cerebral cortexes were less affected [Figure 6].

Comparative analysis of venom exposure of different doses on brain vessels and MGCs activity showed that lower doses (equivalent to 1.0 or 1.5 LD<sub>50</sub>) had no dramatic effect on the brain tissue [Figure 7].

The increased activity of microglia was detected during exposure to the venom. The MGCs became well visible due to higher ATPase activity and, with time, more of these activated MGCs get into direct contact



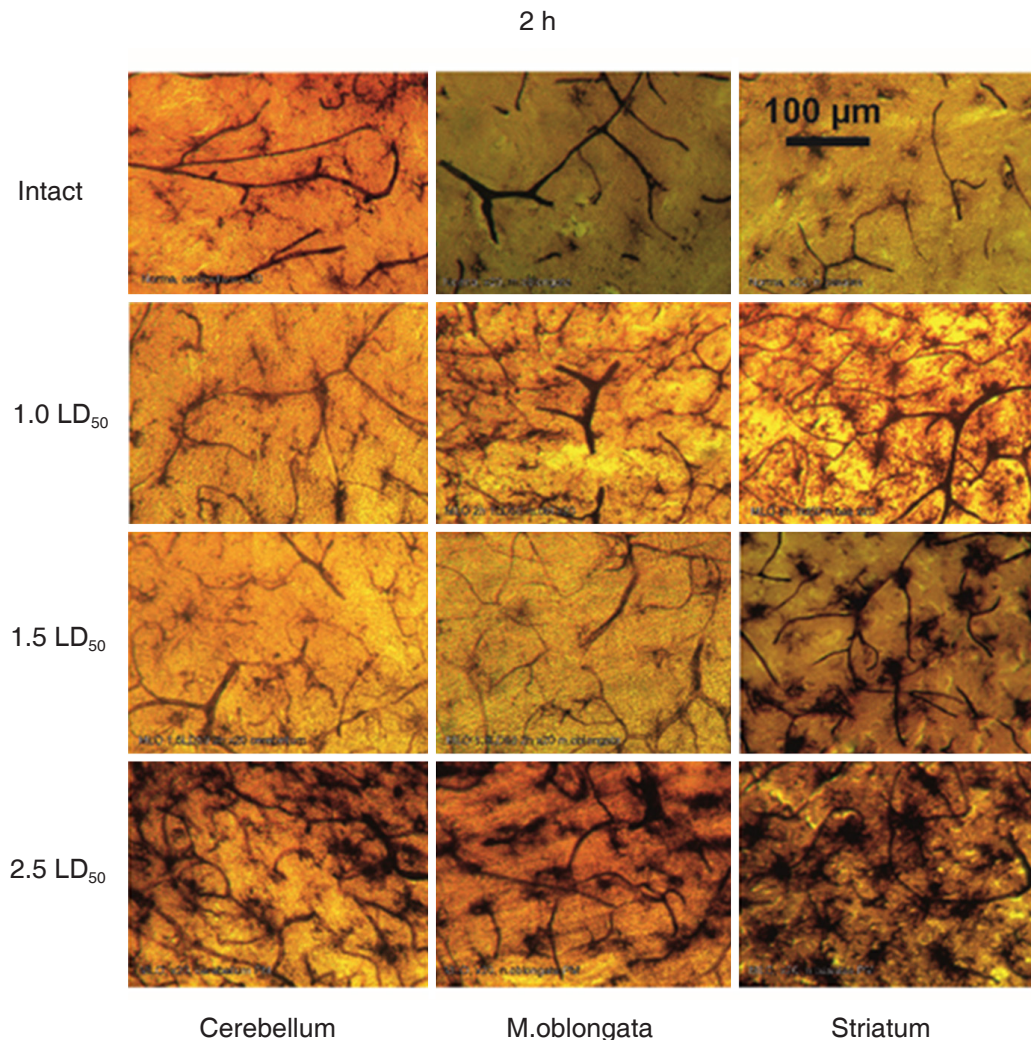


**Figure 6.** Comparative morphology of rat brain sections taken from indicated regions of an intact animal (left column) and from rats exposed to 2.5 LD<sub>50</sub> dose of *Macrovipera lebetina obtusa* after 1 h and 2 h post injection (middle and right column respectively)

with capillaries [Figure 8].

A comparative study of the effects of different doses with the same time of exposition and the same doses for different times of exposition showed that ATPase and MGCs activation are both time- and dose-dependent.

Photomicrographs show that after the shorter exposure and/or smaller dose of MLO venom MGCs have accented ramifications and looked like stellar structures. This indicates a low level of ATPase activation in MGCs and neighboring vessels. In the case of larger dose and/or longer exposure to the venom, MGCs appear as massive dark spots [Figure 9].



**Figure 7.** Morphology of three different areas of the rat brain upon exposure the *Macrovipera lebetina obtusa* venom at 1.0LD<sub>50</sub>, 1.5LD<sub>50</sub>, and 2.5LD<sub>50</sub>, 2 h after injection. Microglial cells activity is markedly elevated and similar only to higher dose 2.5 LD<sub>50</sub> and long period 2 h as well

Changes in the areas, occupied by MGCs show their transformation from stellate to amoeboid shape. This indicates the transition of “primed” MGCs<sup>[25]</sup> into its activated state [Figure 10]. The mean perimeter of activated MGCs was  $705.9 \pm 165.7 \mu\text{m}$  and the mean perimeter of amoeboid MGCs:  $373.4 \pm 55.4 \mu\text{m}$ ,  $P < 0.002$ .

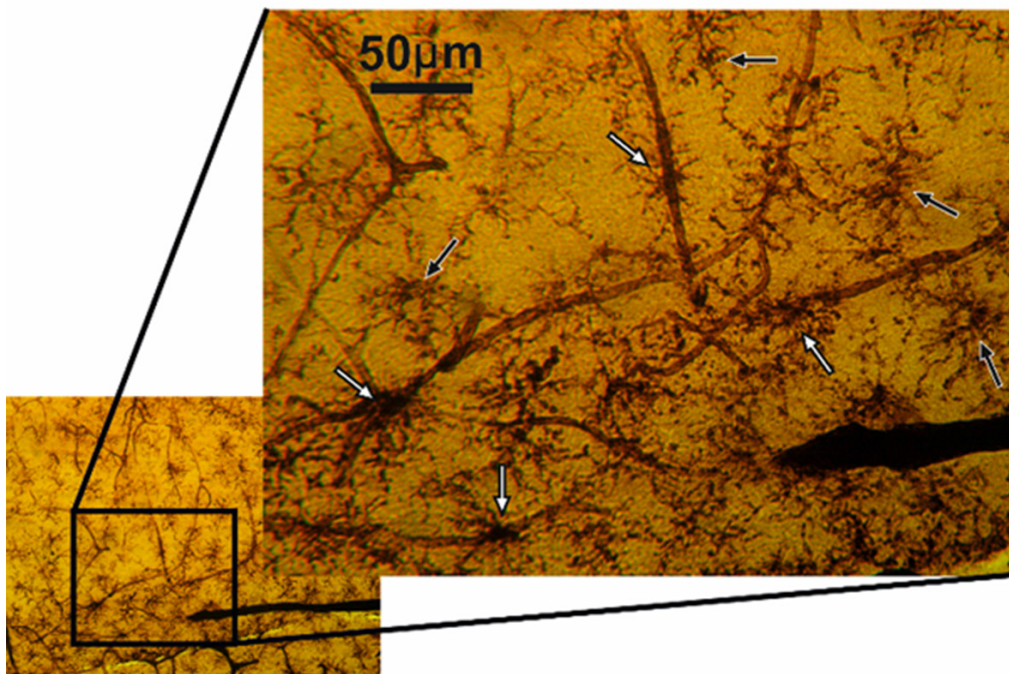
After MLO venom exposure, MGCs show changes similar to changes associated with other pathological processes, such as inflammation and hemorrhagic stroke<sup>[26]</sup>. Activation of MGCs correlates with an increase in ATPase activity. Specifically, an increase of venom dose, and/or the time of exposure results in the higher intensity of MGCs staining. To quantify the changes identified in photomicrographs the intensity of MGCs staining was examined. Collected images were processed by ImageJ to evaluate the corresponding level of MGCs activation [Figure 11].

The values shown are the percentile of the total area of the image occupied by the pixels representing glial cells and vessels with high levels of ATPase activity. ATPase activity of damaged vessels and activated MGCs lead to development of darker staining and indicates action of venom.

#### Calculation of the quantitative parameters of MGCs' size and form

To quantify the changes identified in microphotographs we delineated the MGCs. It is important to mention



Dose 2.5 LD<sub>50</sub>, 1 h

**Figure 8.** Hippocampal region of rat brain after 1 h of *Macrovipera lebetina obtusa* venom injection at 2.5 LD<sub>50</sub> dose. The black arrows show microglial cells in the middle of neighbor vessels area, white arrows show MGC in contact with capillaries

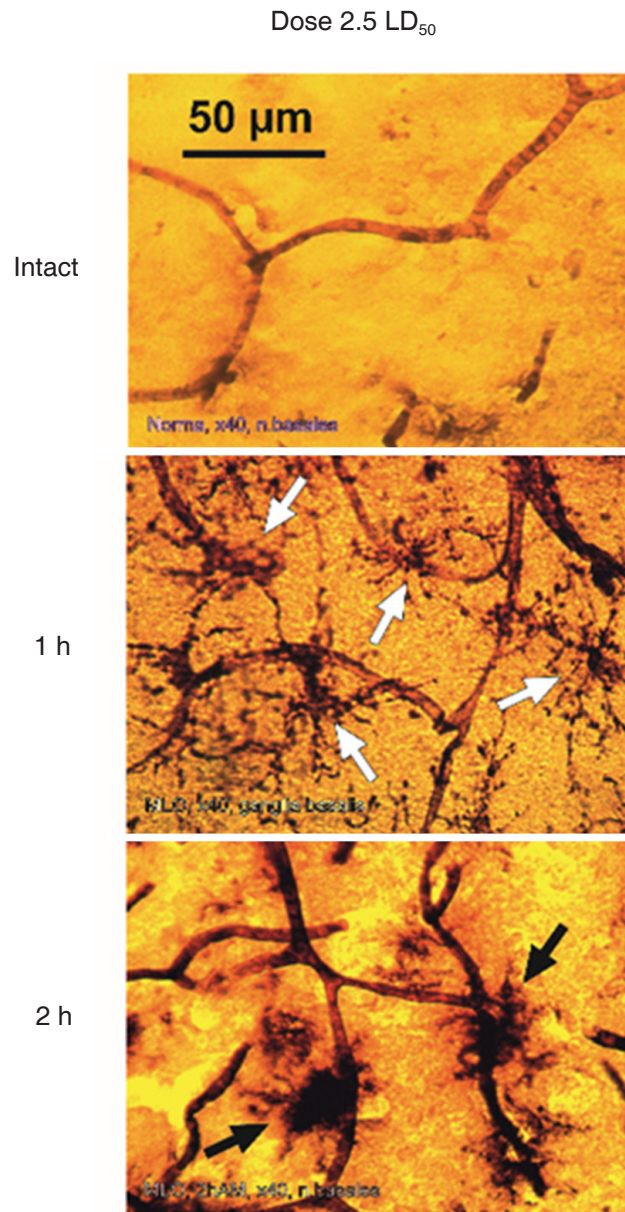
that we were not working with the real contours of cells, but rather with ATP-ase enzyme activity visualized as dark shapes of activated MGC. Reduction of the area, occupied by MGCs shows their transformation from stellar to amoeboid shape associated with corresponding stages of activity [Table 2]. The results demonstrate that general area occupied by stellar MGCs and amoeboid MGCs is  $22334 \pm 5092$  and  $6628 \pm 370$  relative units respectively, i.e., area of amoeboid MGCs shrinks approximately 3 times when the target is reached.

In the case of MLO venom action, MGCs show changes similar to any other kind of pathological processes, including inflammation and hemorrhagic stroke as well<sup>[26]</sup>.

## DISCUSSION

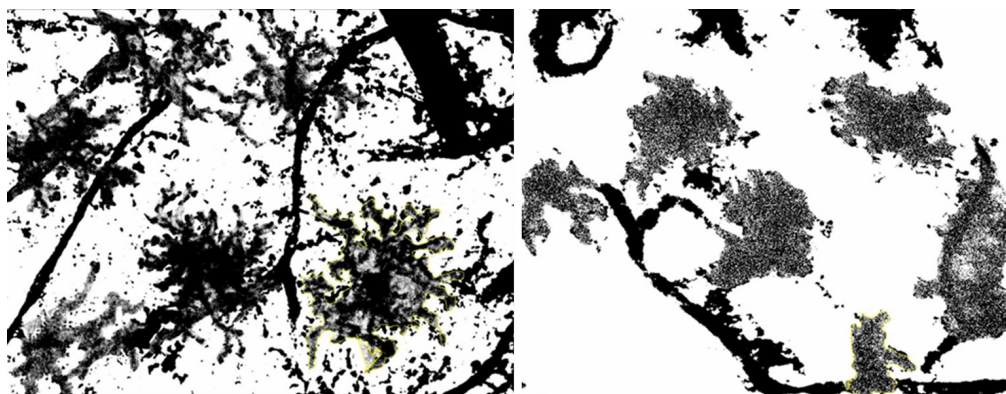
One of the major effects of MLO venom on brain tissue is the induction of hemorrhage. Metalloproteinases destroy basal membrane of blood vessels on which the endothelial cells are anchored. However, the targets of the MLO venom enzymes are not only the basement membrane proteins - collagen IV and laminin, but also proteins of endothelial cells - integrins and cadherins. In addition, when the destruction of the capillary bed begins, many penetrated substances also damage brain tissue. Specifically, fibrinogen and damaged by venom PLA<sub>2</sub> red blood cells get in the brain. Many different derivatives of damaged membranes such as lysophosphatidylcholine, arachidonic acid, platelet activating factor *etc.* infiltrate the brain tissue as well. In the case of more substantial damage, the infiltration of leukocytes into the brain tissue also occurs<sup>[27]</sup>. Moreover, serine proteases, L- $\alpha$ -amino acid oxidase and a cluster of other physiologically active substances, enter the brain tissue and destruct vital structures<sup>[2,28]</sup>.

During our investigation, we used doses of venom which impact brain tissue markedly enough to cause morphological changes. The maximal experimental dose was equal to 2.5 LD<sub>50</sub>, and rats lived during

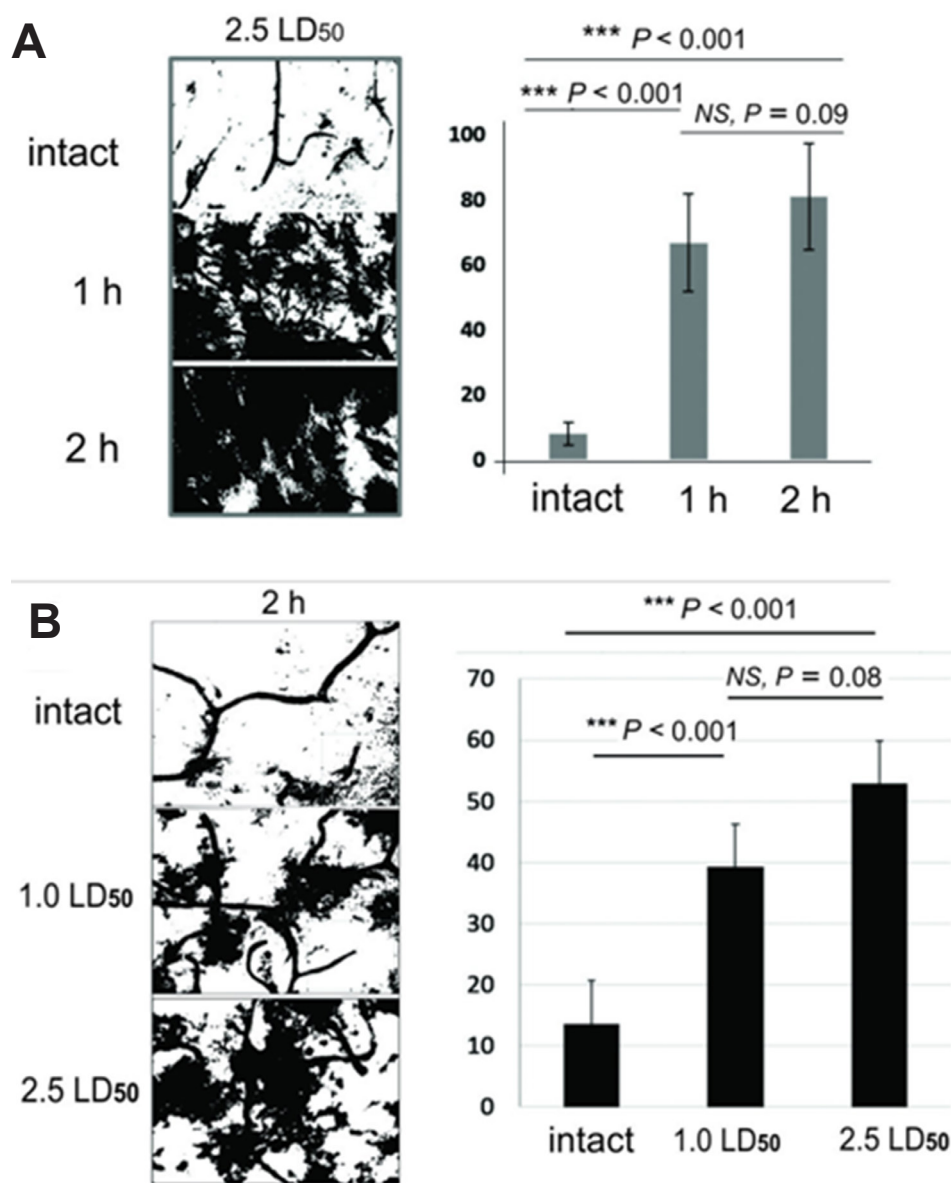


**Figure 9.** Microglial cells (MGCs) activation in striatum after 1 h and 2 h of venom injection. *Macrovipera lebetina obtusa* venom dose 2.5 LD<sub>50</sub>. Activated/primed MGC are shown with white arrows, “proinflammatory state”<sup>[39]</sup> with black arrows

2-2.5 h, and sometimes longer. This gave an opportunity to take the animal brain *antemortem* and avoid *post-mortem* brain changes not associated with the effect of the venom. The method of the histochemical study of microcirculatory bed used in this study is based on the principle of detecting the activity of ATPase in the capillaries leading to deposition of lead sulfide as a contrast agent for light microscopy. However, it can also be used to stain activated microglia. “Resting” MGCs have relatively low metabolism and low level of ATPase activity, so virtually no microglia is detected by staining of the brain of intact animals<sup>[29,30]</sup>. However, analysis of brains of animals exposed to MLO venom clearly identifies activation of MGC in different stages<sup>[31]</sup>. Microglia can be activated in the presence of many damaging factors, for example, proinflammatory stimulation of lipopolysaccharide, ischemia, elevated concentrations of amyloid protein, fibrinogen, thrombin, or ATP, or any other signaling molecule, which induce receptors expression on MGC. Our results corroborate with results of other investigators, which were obtained in both *in vitro* and *in vivo* models of activated microglia<sup>[32]</sup>. There is also data about the activation of microglia in the hippocampus



**Figure 10.** Hippocampal microglial cells (MGCs) transformation during venom exposure. Representative images of MGCs patterns for measurements of perimeter are shown



**Figure 11.** A: Quantification of ATPase activity in rat brain striatum after 2 h of 1.0 LD<sub>50</sub> and 2.5 LD<sub>50</sub> dose; B: quantification of ATPase activity in rat brain hippocampus after 1 h and 2 h of 2.5 LD<sub>50</sub> venom dose envenomation



**Table 2. Calculation of the quantitative parameters of the microglial cells in two groups in the hippocampus of rat brain**

Cell	Six microglia cells, activated				Six microglia cells, final stage			
	Area	Mean	Min	Max	Area	Mean	Min	Max
Rat brain, 1 h after <i>Macrovipera lebetina obtusa</i> injection, hippocampus								
1	28378	61.0	20	121				
2	24150	98.5	3	243				
3	27466	49.5	17	140				
4	17743	99.9	2	235				
5	20302	95.1	0	235				
6	16146	96.8	1	232				
Average	22364	83.5	7	201				
Rat brain, 2 h after MLO injection, hippocampus								
1					7126	86.0	6	228
2					6503	85.0	4	228
3					6810	86.0	4	219
4					6690	85.6	4	214
5					6009	87.6	2	227
6					6631	87.5	4	222
Average					6628	86.3	4	223

in response to acute or chronic stress and in experimental models of ischemia<sup>[33]</sup>. In addition, many studies explored the role of microglia in various models of neurodegenerative diseases such as Alzheimer's, Parkinson's, *etc.*<sup>[34]</sup>. MGCs are continuously scanning surroundings with their "tentacles" in every direction and in the case of detection of damage-associated molecules, stretch their processes in this direction. Based on our data, we presume that the routine scan does not consume much energy and does not lead to increased ATPase activity. However, in the case of envenomation, the MGC receives a signal about damage from astroglial cells<sup>[35]</sup>. It is known that special connexin hemichannels and P2Y metabotropic purine receptors are activated and MGCs begin their movement toward damaged area<sup>[27]</sup>. Such partial or full activation of microglia naturally should lead to activation of enzymatic activity of ATPase and synthesis of new molecules of this enzyme to enable movement. In our investigation, the increased activity of ATPase in brain tissue of venom-exposed animals is detected. Simultaneous visualization of both the microcirculatory bed and activated microglia allows for detection of the distance and position between the capillaries and the MGC in hemorrhagic lesions upon MLO venom exposure. In the early stages of activation, the glial cells have a shape of a spider web and cell bodies are smaller compared with the area occupied by the whole cell<sup>[36]</sup>. These cells are approximately equidistant from nearby capillaries. At later stages of activation, MGCs become more rounded with a small number of "tentacles" and are located very close, or in direct contact with the nearest damaged vessel. The same phenomenon was described in the study of the early stage of multiple sclerosis: the authors observed the interaction of MGCs with capillaries when fibrinogen molecules diffuse from blood to brain tissue<sup>[37]</sup>. Animal studies also demonstrated that during venom exposure period microglia changes its shape and cells accumulate around blood vessels<sup>[32]</sup>.

Obtained data indicate varying degrees of involvement of microglia in the different regions of the brain during intoxication of prey's organism. This could be related to the anatomy of the blood supply paths, and with a specificity of ligands in different brain structures. Thus, the degree of activation of microglia and changes of its form, size, and position are good indicators of hemorrhage-induced cerebrovascular damage. Hemorrhagic effect of MLO venom on the brain tissue of rats is very similar to the hemorrhagic stroke developing in the human brain<sup>[38]</sup>. In some cases, systemic or local (intracerebral) injection of MLO venom may be used for hemorrhagic lesions modeling for experimental needs. Any alteration in brain tissue metabolism related to capillary damage activates microglial cells, which produce certain signaling molecules<sup>[39,40]</sup>. The specific combination of such changes can serve as a diagnostic method to identify development of hemorrhage stroke, thus becoming an important clinical tool.



## DECLARATIONS

### Authors' contributions

Conception, design, supervision, analysis and/or interpretation: Voskanyan A  
 Materials: Darbinyan A, Koshatashyan H, Gevorgyan S  
 Data collection and/or processing: Darbinyan A, Koshatashyan H, Antonyan M  
 Literature review: Voskanyan A, Antonyan M  
 Manuscript writing: Voskanyan A, Karabekyan Z, Arestakesyan H  
 Critical review: Ayvazyan N

### Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

### Financial support and sponsorship

None.

### Conflicts of interest

Authors have no conflicts of interest related to this article. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Copyright

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Review

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# It takes two: potential therapies and insights involving microglia and macrophages in glioblastoma

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## Abstract

Microglia and macrophages, two myeloid cell lineages with different origins, make up the majority of immune cells present in glioblastoma (GBM). However, much of the literature does not distinguish between microglia and macrophages, despite a growing body of evidence that demonstrates key structural and functional differences between the cell types. Furthermore, the current M1/M2 paradigm used to sub-classify microglia and macrophages has proven to be incomplete at best, with the growing amount of *in vivo* and genomic data incompatible with this dichotomy. Finally, a number of studies have already established that in the setting of the GBM tumor microenvironment, both microglia and macrophages are complicit in tumor progression. This review highlights the differences between microglia and macrophages, particularly in the context of GBM, and discusses at length several potential therapeutic strategies made possible by understanding specific pro-tumor and anti-tumor pathways in these myeloid populations. Ultimately, investigating the differences between microglia and macrophages offers insight into the progression of GBM, its marked resistance to current immunotherapy regimens, and future directions for new treatment modalities.

**Keywords:** Glioblastoma, cancer, immunotherapy, myeloid, microglia, macrophages, pro-tumor, anti-tumor, immunosuppression

## INTRODUCTION

The advent of immunotherapy as a viable cancer treatment option has resulted in the rapid emergence of new therapeutic strategies, with immune checkpoint inhibitors (ICI) serving as the cornerstone for



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mounting anti-tumor immune responses against several types of cancers like non-small-cell lung cancer, bladder cancer, and advanced-stage melanoma<sup>[1-5]</sup>. Intrinsic to ICI-based therapies, particularly those blocking cytotoxic T lymphocyte antigen 4 and programmed cell death protein 1 (PD-1), is the recruitment of CD8<sup>+</sup> T cells from tumor infiltrating lymphocytes (TILs) that are normally immunosuppressed in the tumor microenvironment (TME)<sup>[6]</sup>. However, certain cancers have remained resistant to current immunotherapeutic strategies and are considered “cold tumors”; the recent phase III CheckMate 143 trial involving nivolumab, an anti-PD-1 drug, failed to meet its primary endpoint of improved overall survival in patients with glioblastoma (GBM)<sup>[7,8]</sup>.

GBM is the most aggressive intrinsic brain tumor, with median overall survival ranging from 12 to 15 months in patients who receive current standard of care<sup>[9]</sup>. While ICI has shown some promise in preclinical GBM models - particularly in combination with radiation therapy - emerging studies support the idea that GBM is a cold tumor, meaning that it shows more resistance to anti-PD-1 when compared to other hot tumors like melanoma that respond to ICI therapies; in regards to this difference in response, GBM appears to have a (1) T cell population with high expression of exhaustion markers such as lymphocyte-activation gene 3 (LAG3/CD223) and T-cell immunoglobulin mucin 3 (TIM3); (2) relatively few TILs; and (3) a high volume of myeloid cells (i.e., microglia and macrophages) that make up about 30%-40% of the tumor cell population<sup>[10,11]</sup>.

As such, there is interest in exploring additional candidates for immune cell reactivation beyond lymphocytes, particularly within the myeloid population<sup>[12]</sup>. Along this line, several studies in the last decade have revealed immunosuppressive and pro-tumor characteristics in microglia and macrophages within the TME, resulting in a growing interest in viewing these myeloid cells as potential therapeutic targets<sup>[12-14]</sup>. It should be noted that while granulocytic or monocytic myeloid-derived suppressor cells (MDSCs) are also considered to be a part of the myeloid compartment, there is limited data regarding specific markers that easily distinguish MDSCs from monocytes and will therefore not be addressed further as a distinct population from microglia and macrophages at this time<sup>[15]</sup>.

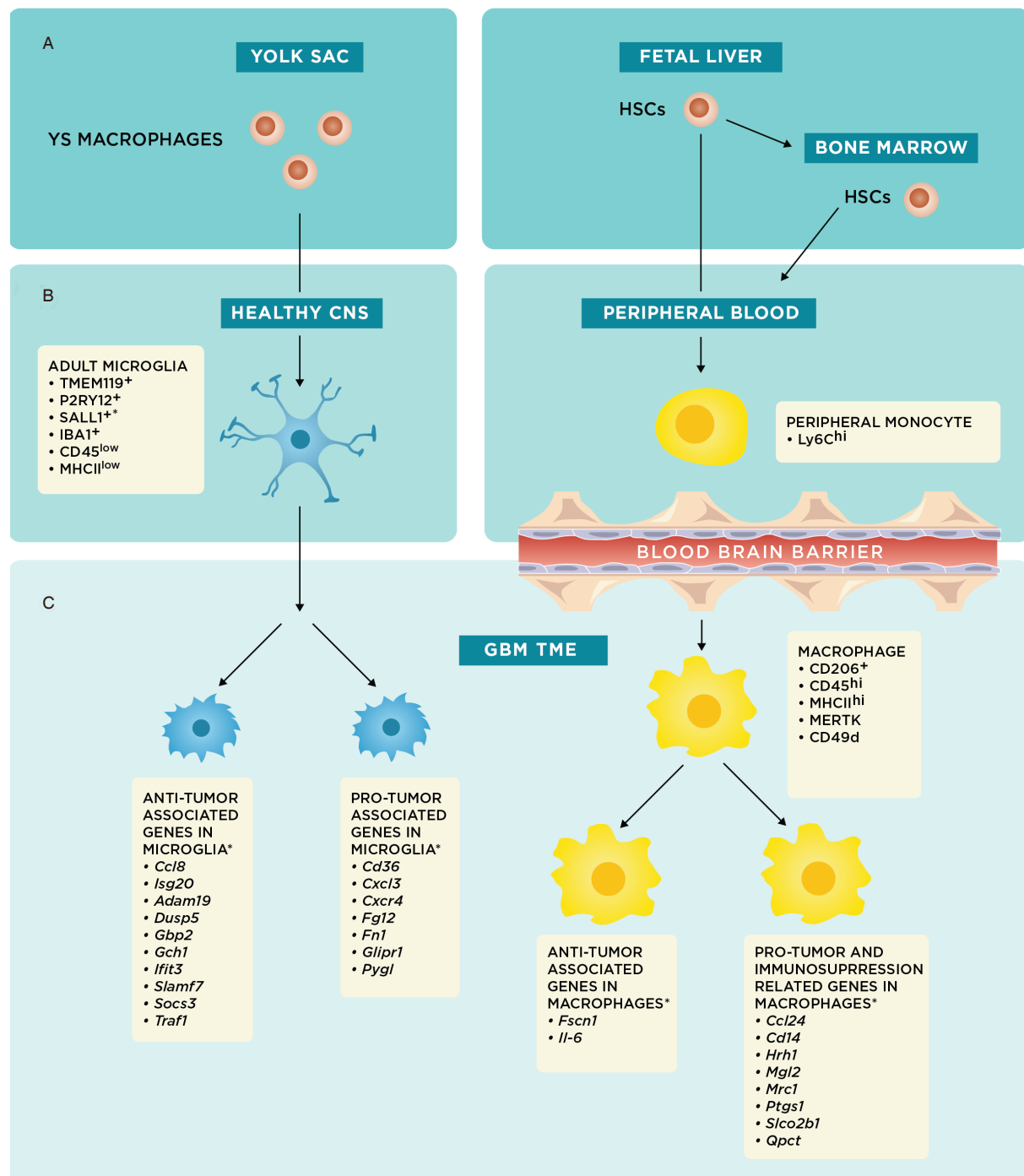
As such, the purpose of this review is to distinguish the structural and functional differences of microglia and macrophages in the context of the TME of GBM, expand upon the roles of microglia and macrophages in GBM progression and invasion, and discuss current and potential treatment strategies involving these two cell populations.

### **Macrophages and microglia: similar but distinct populations**

Historically, microglia and macrophages have generally been considered interchangeable in the TME, with the former functionally described as the macrophages of the central nervous system (CNS). While both cell types have shared immunologic functions, including phagocytosis of microorganisms and cell debris with subsequent antigen presentation to lymphocytes<sup>[16]</sup>, the advent of genome-wide microarray analyses and detection of specific cellular markers have phenotypically distinguished these cell populations [Figure 1]<sup>[17,18]</sup>.

Moreover, embryological studies like those from Janossy *et al.*<sup>[19]</sup> and Ginhoux *et al.*<sup>[20]</sup> have shown that microglia and macrophages come from distinct embryological origins [Figure 1A]<sup>[13]</sup>. Microglia, which are endemic to the CNS, come from yolk sac progenitors and migrate to the brain early in development<sup>[19]</sup>. The prevailing thought is that microglial populations are enduring and maintain their numbers primarily through local self-renewal<sup>[13,20]</sup>. Recent fluorescent fate-mapping studies from Tay *et al.*<sup>[21]</sup> support this model and also suggest that this self-renewal process is stochastic in the normal steady-state, independent of bone marrow (BM) input, and dependent upon brain geography and inflammatory status.

However, during states of CNS inflammation, BM-derived macrophages can be drawn into the CNS



**Figure 1.** \*These markers and/or genes have been described in detail mostly in preclinical mouse models as described in this review. Origins and specific cellular markers and genes of microglia and macrophages. (A) Schematic summarizing the embryonic origins of microglia and macrophages from the yolk sac (YS) and fetal liver, respectively; (B) YS macrophages migrate to the central nervous system (CNS) early in embryonic development and remain in the brain as tissue resident macrophages, or microglia. Fetal liver and bone marrow hematopoietic stem cells (HSCs) mature into monocytes and enter the peripheral blood<sup>[13]</sup>; and (C) during states of CNS inflammation, particularly in the context of glioblastoma (GBM) tumor microenvironment (TME), microglia and macrophages experience shifts in phenotype; the underlying genetic changes are schematically represented here to show pro-tumor or anti-tumor associated genes<sup>[12]</sup>. In reality, there are most likely microglial and macrophage populations that have concurrent expression of anti-tumor and pro-tumor genes



from the periphery by the same chemokines that increase permeability of the blood-brain-barrier<sup>[22]</sup>. As a result, while microglia will always be present in brain tissue regardless of inflammatory status, peripheral macrophages should only be present in significant numbers during periods of inflammation<sup>[22]</sup>.

While embryological studies clearly reveal that these two populations have distinct origins, the phenotypic differences between microglia and macrophages have often been overlooked. During recruitment of peripheral monocytes under inflammatory conditions in both the neonatal and adult brain, Ling<sup>[23]</sup> showed that peripheral monocytes have the potential to histologically differentiate into microglia-appearing cells within the CNS. This finding perpetuated the notion that circulating blood monocytes could act as microglial progenitors that replenished microglial populations<sup>[24]</sup>. It should be stressed that because these original findings were based on histological morphology, the conclusions drawn on their functional differences were limited; moreover, using histologic structural characteristics on microscopy to differentiate microglia and macrophages is unreliable since both cell types have morphologies that are plastic and inconsistent<sup>[25,26]</sup>. These older studies also could not take advantage of the results from more recent bulk-RNA sequencing studies, which have since elucidated specific cellular markers for microglia, such as CD45<sup>low</sup>, major histocompatibility complex II (MHCII)<sup>low</sup>, transmembrane protein 119, P2Y purinoceptor 12, IBA1, and Sal-like protein 1 [Figure 1B]<sup>[27-29]</sup>. Similarly, macrophages have their own specific cellular markers, including CD45<sup>hi</sup>, MHCII<sup>hi</sup>, CD49d, CD206, and *MER* receptor tyrosine kinase (MERTK) [Figure 1C]<sup>[30-35]</sup>.

Beyond cellular markers, microglia have characteristics that are functionally distinct from macrophages. While microglia are considered the resident immune cells of the CNS and perform roles similar to macrophages including phagocytosis and antigen presentation, they are also thought to have additional roles in homeostasis such as secretion of neurotrophic factors that are essential for both normal maintenance and response to pathological conditions<sup>[36]</sup>. As a key component to normal parenchymal surveillance, microglia are mobile within their own distinct territories and completely scan the brain parenchyma several times a day<sup>[37]</sup>. While scanning, microglia are sensitive to ATP, potassium, and purinoceptor inhibitors, and as such can detect neuronal cell death or other pathological features with high acuity<sup>[38,39]</sup>. Upon activation, they convert to an amoeboid phenotype and act similarly to macrophages with a high metabolic rate, rapidly migrating to the source lesion and secreting IL-6, IL-1 $\beta$ , and TNF $\alpha$  before phagocytosing as needed<sup>[40]</sup>.

Of note, while tissue-specific macrophages can be found in other organs outside of the CNS, microglia are special in part due to the brain's privileged status behind the blood-brain barrier (BBB); after embryonic migration, they remain and exert their effect only in their original tissue with minimal interaction with other systems<sup>[41]</sup>. This is in contrast to other tissue-specific macrophages, like Langerhans cells, which are epidermal-specific macrophages that have the capacity to migrate to peripheral lymph nodes upon activation<sup>[19]</sup>, or intestinal macrophages, which act locally but rely less upon self-renewal and more upon recruitment of circulating macrophages to maintain their numbers<sup>[42]</sup>.

In the context of this review however, the strongest rationale for viewing microglia and macrophages as distinct populations emanates from their functional differences in the context of the TME, specifically in that of GBM. In the CNS, mild or moderate inflammation leads to the protective function of microglia as outlined above and features minimal interaction with peripheral macrophages. However, more intense acute injury or chronic inflammatory states - as experienced in GBM - can lead to neurotoxic and tumor-promoting activation of microglia, recruitment of peripheral macrophages, and subsequent immunosuppression<sup>[41]</sup>. More specifically, chemokines released in the TME attract peripherally derived macrophages, which then migrate into the brain through the BBB and express anti-inflammatory cytokines that attenuate the recruitment and aggregation of pro-inflammatory leukocytes (e.g., additional microglia or neutrophils)<sup>[35]</sup>. The complexity of the GBM TME with this consequent anti-inflammatory attenuation ultimately contributes to pathology and promotes gliomagenesis<sup>[43]</sup>.

### M1/M2: an outdated paradigm

Within the GBM TME, microglia and macrophages have classically been subdivided into M1 and M2 phenotypes to characterize them as either having anti-tumor or tumor-promoting (pro-tumor) properties, respectively. The M1/M2 dichotomy was first discussed by Mills *et al.*<sup>[44]</sup> as a way to distinguish the phenotypic predilections of macrophages from the perspective of Th1 and Th2 lineages in CD4<sup>+</sup> T cells; they proposed that M1 refer to macrophages with Th1 backgrounds that tended to produce inflammatory induced nitric-oxide species (iNOS), while M2 would refer to Th2 derived macrophages that produced more cell-division stimulating polyamines, like ornithine. In short, the original M1/M2 terminology sought to extrapolate the same phenotypic dichotomy assigned to T-helper cells (Th1/Th2) to macrophages<sup>[44]</sup>. However, later research elucidated further phenotypic pathways for macrophages related to other cytokines and factors (e.g., IL-10, TGF- $\beta$ ) that made the extrapolation from the initial binary Th1/Th2 characterization less robust<sup>[45]</sup>. As a result, Mantovani *et al.*<sup>[46]</sup> proposed the conversion of the M1/M2 dichotomy into a continuum, with M1 and M2 representing two opposite poles of immune function.

In this vein, M1 macrophages, or classically activated macrophages, are typically noted as inducing prototypic inflammatory (pro-inflammatory) responses, while M2 macrophages, or alternatively activated macrophages, are those with antagonism of normal inflammatory (anti-inflammatory) responses<sup>[47,48]</sup>. More specifically, M2 has been further divided into subtypes; M2a correlates to Th2 responses, type II inflammation, and pathogen elimination. M2b correlates to Th2 activation and immunoregulation. Finally, M2c correlates to immunoregulation, matrix deposition, and tissue remodeling<sup>[46]</sup>. Ultimately, the popularity of using the M1/M2 paradigm in studies came from its simplicity; by mirroring the nomenclature used for the Th1/Th2 phenotypes, M1/M2 provided a simple and easy to conceptualize model to understand immunosuppressed myeloid populations<sup>[46]</sup>.

However, while the M1/M2 framework was designed to be a simplified operational concept that provided foundational language to a rapidly growing field<sup>[48]</sup>, it has since been used erroneously in much of the literature as a solid classification scheme for macrophages, and to an increasingly greater extent, microglia. From a generalized view, using M1/M2 as a classification system is problematic for a variety of reasons. First, the vast majority of data supporting the system comes from *in vitro* studies that have not been reliably recapitulated *in vivo*<sup>[49]</sup>. These concepts rarely translate to systemic models, as *in vitro* systems have limited engagement with larger systemic variables beyond characteristics of cell maturation, adhesion, and cytokine production<sup>[50]</sup>. More specifically, macrophages *in vitro* versus macrophages *in vivo* have been documented to have different morphologies, functions, and expression of specific cellular markers<sup>[49]</sup>.

Beyond experimental inconsistencies, the M1/M2 phenotypes are also outdated in the sense that their original formulation predated the significant new body of genomics research that has emerged in the last 15 years. Genome-wide microarray analysis of both glioma-associated microglia and macrophages in GL261 murine gliomas by Szulzewsky *et al.*<sup>[17]</sup> have shown that both cell types have expression profiles that do not fit within any previously documented M1/M2 classification scheme<sup>[51]</sup>. Indeed, these myeloid populations only had partial overlap with previously documented M1/M2 phenotypes, with 59.6% of genes that were significantly upregulated (261/438 analyzed) not characterized as either M1 or M2; this indicates that there is far more complexity than the M1/M2 label can provide, at least from a genomics standpoint<sup>[17]</sup>. Of note, some of the genes identified outside of the classic M1/M2 phenotype were associated with angiogenesis (*Vegfa*, *Hgf*), suppression of immunity (*Arg1*, *Tgfb3*), and tumor invasion (*Mmp2*, *Mmp14*, *Ctgf*) in mouse models<sup>[17]</sup>.

In the context of this review, the M1/M2 classification is further problematic when applied to microglia. The original nomenclature for the M1/M2 classification came from studying macrophages and a significant portion of the current literature has merely transposed this M1/M2 nomenclature to microglia without respect to the differences between these two myeloid populations<sup>[45]</sup>. As discussed previously, however, it

was also only recently that we have come to recognize the numerous differences between microglia and macrophages in terms of structure, function, and expression of tumor-related pathways<sup>[17,18]</sup>. As a result, while the M1/M2 nomenclature for macrophages has issues with oversimplification, the use of said classification scheme for microglia may be simply inaccurate. Importantly, a variety of genome-wide expression studies of microglia in a variety of disease state models, including generalized inflammatory states<sup>[18]</sup>, amyotrophic lateral sclerosis (ALS)<sup>[49]</sup>, autoimmune encephalomyelitis<sup>[52]</sup>, Alzheimer's disease<sup>[53]</sup>, traumatic brain injury<sup>[54]</sup>, and GBM<sup>[55]</sup> all showed no clear evidence of true M1/M2 differentiation among microglia and instead mostly showed that they simultaneously express both M1 and M2 phenotypic markers.

Furthermore, it is difficult to classify microglia along the M1/M2 continuum, especially in the context of GBM, since activated microglia in the TME have several more functions that do not fit into the classic functional categories associated with M1/M2 in macrophages. For example, while the M2 phenotype for macrophages has largely been regarded as being immunosuppressed - specifically in the context of increased trophic polyamines - the M2 subtype in microglia may actually have a more active pro-tumor role that supports gliomagenesis and invasion<sup>[22,46,47,56-60]</sup>.

As a result, we recognize the equivocal and limited nature of using M1/M2 as a classification scheme and will be utilizing a more flexible paradigm to organize our discussion of microglia and macrophages, specifically within the context of the TME in GBM. To address these differences as well as move away from the M1/M2 nomenclature, we focus on specific markers and pathways present on microglia or macrophages and designate these molecular targets as having either anti-tumor or pro-tumor/immunosuppressive characteristics [Figure 2].

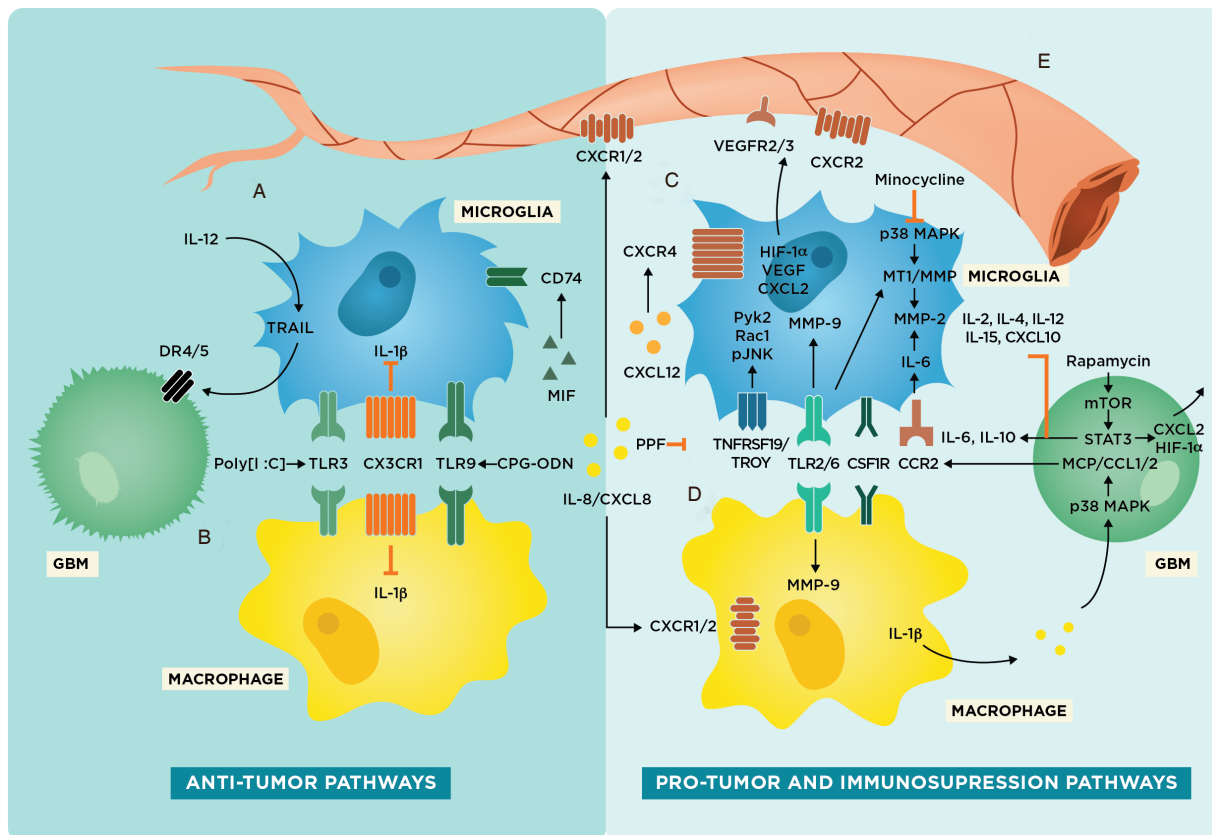
### **Therapeutic strategies involving glioma-associated microglia and macrophages in the GBM TME**

Several preclinical and clinical studies have already examined the efficacy of targeting glioma-associated microglia and macrophages for anti-tumor therapy [Table 1]. In general, there are two main strategies for treatment: inhibition of tumor-promoting microglia and macrophages [Figure 2C and D] or upregulation of anti-tumor receptors and cytokines in microglia and macrophages [Figure 2A and B]<sup>[61,62]</sup>. It should be noted that while there are some receptors and resultant cascades that are present in both microglia and macrophages, there are also a variety of signaling pathways unique to either microglia or macrophages, which lends credence to the idea that these cell types have distinct roles in the TME.

#### *Activation of the intrinsic anti-tumor properties of microglia and macrophages*

First, while much of the scientific literature corroborates a story of microglia and macrophages having mostly a pro-tumor or immunosuppressive role in the GBM TME, there is some evidence that microglia and macrophages have intrinsic anti-tumor properties as well. An *in vitro* study by Hwang *et al.*<sup>[63]</sup> demonstrated that microglia conditioned culture medium (MCM) promotes apoptosis of glioma cells, with additional cytotoxic effect when exposing microglial cells to lipopolysaccharides (LPS) or IFN $\gamma$ . Moreover, this effect was glioma-specific, without unwanted astrocyte cytotoxicity. Proteomic analysis of the MCM revealed LPS- and IFN $\gamma$ -related proteins along with markedly elevated expression of cathepsin proteases - particularly cathepsin B. When cathepsin B was suppressed, glioma-apoptosis was no longer observed, indicating this protein's importance in microglial anti-tumor function<sup>[63]</sup>.

A similar study by Kees *et al.*<sup>[64]</sup> examined toll-like receptor 3 (TLR3) and its agonist, polyinosinic-polycytidylic acid [poly(I:C)], on both microglia and macrophages. The activation of TLR3 on these myeloid cells resulted in the secretion of glioma specific toxic soluble factors in co-culture with GBM cells [Figure 2A and B]. Of note, a similar phenomenon involving poly(I:C)-induced TLR3 activation in dendritic cells demonstrated by Garzon-Muvdi *et al.*<sup>[65]</sup> showed anti-tumor effect. Likewise, TLR9 has also



**Figure 2.** Glioma-associated microglia and macrophage pathways in the tumor microenvironment including potential therapeutic targets. Schematic representing anti-tumor and pro-tumor pathways in microglia. (A) Intrinsic anti-tumor pathways present in microglia involving TRAIL, IL-1 $\beta$  inhibition, and CD74 upregulation; (B) Intrinsic anti-tumor pathways present in macrophages involving IL-1 $\beta$  inhibition. toll-like receptor 3 (TLR3), CX3CR1, and TLR9 are both present on both microglia and macrophages; (C) pro-tumor or tumor-progressive pathways in microglia that are associated with increased gliomagenesis and invasion; (D) pro-tumor and immunosuppressing pathways present in macrophages that result in decreased immune response against glioblastoma (GBM); and (E) tumor angiogenesis and vasculogenesis pathways involving microglia and macrophages

been implicated in anti-tumoral pathways. Several pre-clinical trials have shown that local treatment with oligodeoxynucleotides containing CpG motifs (CpG-ODN) have strong immunostimulatory effects and activate TLR9 in both microglia and macrophages<sup>[66]</sup>; in a murine glioma *in vivo* study by Carpentier *et al.*<sup>[67]</sup>, the use of CpG-ODN resulted in decreased tumor size without toxicity to brain parenchyma [Figure 2A and B]. Unfortunately, follow-up studies in humans including a phase II trial did not show significant progression free survival or radiological response in patients treated with CpG-ODN<sup>[68,69]</sup>.

More promisingly, another study demonstrated the importance of IL-12 in the modulation of microglial anti-tumor activity in mouse models. Using recombinant adenovirus-carrying IL-12 (rAAV2/IL-12), Chiu *et al.*<sup>[70]</sup> demonstrated that IL-12 resulted in increased activation of microglia as demonstrated by increased expression of ED1 and tumor necrosis factor-related apoptosis-inducing ligand; *in vitro*, IL-12 exposure also resulted in microglial-mediated apoptosis of GBM cells through DR4/5 binding<sup>[70,71]</sup>. In a follow-up study, they observed a similar effect *in vivo*, with murine GBM models treated with IL-12 exhibiting greater infiltration of activated microglial cells within the tumor mass. Additionally, IL-12 treated mice had significantly reduced tumor volume and increased survival compared to non-treated tumor control groups [Figure 2A]<sup>[70]</sup>.

Finally, Zeiner *et al.*<sup>[72]</sup> found that GBM has high expression of macrophage migration inhibitory factor,

**Table 1. Summary of molecular targets for myeloid interactions with glioblastoma (GBM)** Table summarizing receptors and ligands on microglia, macrophages, and glioma cells that are present in the context of GBM tumor microenvironment, along with their interactions with each other in preclinical and clinical investigations

Molecular targets involved with tumor progression in GBM		Normal function	Aberrant function in tumors	Preclinical studies
Chemokine (C-X-C motif) ligand 2 (CXCL2)- from microglia, macrophages, gliomas	Chemokine (C-X-C motif) receptor 2 (CXCR2)- on endothelial cells	Chemoattractant for neutrophils	Angiogenesis	Brandenburg 2015 <sup>[97]</sup> - CXCL2 was upregulated and stronger than VEGF <i>in vitro</i> -blocking CXCL2-CXCR2 resulted in diminished glioma sizes - <i>in vivo</i> deletion of microglia and macrophages decreased vessel density by 50%
CX3CL1- from neurons	CX3CR1- on microglia and macrophages	Chemokine mediation of immune response	Deletion on macrophages promotes gliomagenesis; accumulation of inflammatory myocytes	Feng 2015 <sup>[79]</sup> - Deletion of Cx3cr1 from microglia and macrophages leads to increased tumor incidence and shorter survival times
Chemokine (C-C motif) ligand 2 (CCL2)- from microglia and GBM	Chemokine (C-C motif) receptor 2 (CCR2)- on microglia	Chemoattractant for microglia	Recruitment of microglia to tumor site; IL-6 positive feedback cycle for inflammation	Carvalho da Fonseca <sup>[62]</sup> , Zhang 2012 <sup>[80]</sup> - CCL2 was found to be produced by GBM - <i>in vitro</i> glioma lines displayed increased invasion of extracellular collagen matrices when co-cultured with CCR2 expressing microglia
CXCL12, from TME	CXCR4- on microglia	Proinflammatory chemokine signaling	Vasculogenesis in radiation-resistant gliomas	Tabouret 2015 <sup>[98]</sup> - CXCR4 blockade in GBM implants lead to decreased VEGF and Hif1a expression
HiF1α- from microglia + GBM		Angiogenesis	Angiogenesis	Tabouret 2015 <sup>[98]</sup> , Brandenburg 2015 <sup>[97]</sup> - Decreased Hif1a expression lead to less angiogenesis and smaller tumor size
VEGF- from microglia, macrophages	VEGFR-2/3- on endothelial cells	Angiogenesis	Angiogenesis	Tabouret 2015 <sup>[98]</sup> , Brandenburg 2015 <sup>[97]</sup> - Decreased VEGF expression lead to less angiogenesis and smaller tumor size
CpG DNA	Toll-like receptor 9 (TLR9) on microglia and macrophages	Innate Immune Response	Gliomagenesis	Carpentier 2010 <sup>[67]</sup> - Phase II clinical trial investigating CpG as a therapeutic did not find a significant improvement in progression-free survival, though a few long-term survivors suggest potential benefit
CXCL8/IL8 from GBM, microglia, macrophages	CXCR1, CXCR2, on endothelial cells, macrophages, and microglia	Neutrophil chemotaxis, angiogenesis	Gliomagenesis, chemoresistance, invasion, angiogenesis	Waugh 2008 <sup>[73]</sup> , Brandenburg 2015 <sup>[97]</sup> - Blocking CXCR2 resulted in considerably diminished glioma sizes
Membrane type 1 metalloprotease (MT1-MMP)	Pro-MMP2 to MMP2	Extracellular matrix breakdown	Invasion	Markovic 2011 <sup>[83]</sup> - Oral minocycline administration greatly reduced glioma growth in orthotopically implanted mice - MT1-MMP was decreased in treated mice and highly upregulated in untreated mice
Colony stimulating factor 1 (CSF1)	Colony stimulating factor 1 receptor (CSF1R) - on macrophages and microglia	Production, differentiation, and function of macrophages and microglia	Gliomagenesis and immunosuppression	Pyonteck 2013 <sup>[76]</sup> - The brain-penetrant CSF1R inhibitor BLZ945 resulted in increased expression of anti-tumor responses in glioma associated macrophages and resulted in decreased intracranial growth of patient-derived glioma xenografts in mice Yan 2017 <sup>[77]</sup> - Combination therapy with CSF1R inhibitor PLX3397 and tyrosine kinase inhibitors dovitinib and vatalanib resulted in increased survival in mice glioma models

VEGF: vascular endothelial growth factor



which can bind to the receptor CD74 on glioma-associated microglia. Interestingly, they found a positive correlation between CD74-positive glioma-associated microglia and patient survival, indicating anti-tumoral characterization of this marker. This positive prognostic factor offers a potential area of exploration into pathways involving CD74 to further elucidate candidate receptors or cytokines for encouraging microglial recruitment for anti-GBM response.

#### *Inhibition of pro-tumor functions of microglia and macrophages*

There are also several strategies that aim to inhibit pro-tumor or reactivate immunosuppressive pathways in microglia and macrophages. Interleukin 8 [IL-8 or chemokine (C-X-C motif) ligand 8, CXCL8] has been implicated in several tumorigenic pathways, most pronouncedly *via* its binding CXCR1/2 on endothelial cells and macrophages; this has been associated with tumor growth and chemoresistance, increased invasion, and tumor angiogenesis<sup>[73,74]</sup>. Furthermore, increased presence of IL-8 has been found in the TME of GBM along with upregulation of its receptors in macrophages and endothelial cells [Figure 2D and E]<sup>[73]</sup>. A follow-up study by Infanger *et al.*<sup>[75]</sup> demonstrated similar findings, with IL-8 linked to maintenance and growth of GBM cancer stem-like cells. In the same investigation, they found that CXCR2 silencing reversed the tumor-promoting effects of endothelial cells *in vivo*, demonstrating the potential therapeutic benefit of inhibiting IL-8 signaling for anti-tumor response.

Further work in understanding the TME and its impact on glioma-associated macrophages include studies on BLZ945 and PLX3397: inhibitors of colony stimulating factor-1 receptor (CSF1R). Contrary to their original hypothesis that CSF1R inhibition would lead to tumor inhibition through global depletion of tumor-associated microglia and macrophages, they actually achieved tumor inhibition in xenograft mouse models through enhanced survival and promotion of tumor-associated macrophages that demonstrated antitumor properties<sup>[76,77]</sup>. Specifically, when Pyonteck *et al.*<sup>[76]</sup> used BLZ945 to inhibit CSF1R in mice, they reported shifts in the gene signatures of macrophage populations away from pro-tumor/immunosuppressive phenotypes with consequent inhibition of GBM progression, *in vivo*. However, continued use of CSF1R inhibitors resulted in acquired resistance to further CSF1R inhibition in GBM mouse models<sup>[78]</sup>. Therefore, a follow-up study by Yan *et al.*<sup>[77]</sup>, focused on using a combinatorial approach with PLX3397 (another potent CSF1R inhibitor) and the tyrosine kinase inhibitors dovitinib or vatalanib; this combination therapy demonstrated significant and lasting reduction in tumor volume compared to PLX3397 alone, indicating that these anti-tumor macrophages rendered glioma cells more sensitive to treatment.

Interestingly, while exposure to PLX3397 preserved macrophage density and resulted in a phenotypic shift, non-glioma associated stromal microglia were almost fully depleted. Yet, while this data suggests the preservation and redirection of tumor-associated macrophages to an anti-tumor phenotype coupled with depletion of stromal microglia in surrounding tissues, neither study adequately characterized the true ratio of microglia to macrophages in the surviving tumor-associated milieu, nor were they able to adequately attribute the ratio of anti-tumor cells to that of peripheral macrophages<sup>[77,78]</sup>. As a result, while their results suggest enhancement of anti-tumor tumor-associated macrophages alone, further characterization of both populations of cells in the context of CSF1R inhibition is necessary to accept that assertion without doubt. Regardless, the CSF1R pathway potentially indicates a promising therapeutic avenue for targeting macrophages in the GBM TME<sup>[77]</sup>.

Additionally, *Cx3cr1* knock-out (KO) experiments by Feng *et al.*<sup>[79]</sup>, gave further insight into the complexity of tumor adaptive pathways involving both microglia and macrophages. The ligand for CX3C chemokine receptor 1 (CX3CR1), CX3CL1 (fractalkine), is an important chemokine-signaling protein in the healthy CNS that mediates inflammatory response of both microglia and macrophages, including properties of adhesion and migration [Table 1]. When deleted, *Cx3cr1* KO mice experienced increased gliomagenesis and greater tumor burden [Figure 2A and B]. Interestingly though, there was no effect on microglial migration

in peri-tumoral areas and instead resulted in a significant increase in macrophage recruitment from the periphery and subsequent infiltration. Of key importance is that deletion of *Cx3cr1* in mice saw an increase in IL-1 $\beta$  production from both microglia and macrophages, implicating this receptor in the suppression of IL-1 $\beta$  production<sup>[79]</sup>.

The importance of IL-1 $\beta$  lies in the IL-1 $\beta$ /CCL2/IL-6 interaction between microglia and glioma cells [Figure 2A-C]. Specifically, IL-1 $\beta$  released from both microglia and macrophages activates the p38 mitogen-activated protein kinase (MAPK) pathway in glioma cells, which in turn results in increased expression of CCL2-the agonist for CCR2 on microglia [Table 1]<sup>[80]</sup>. This results in an increase in microglial production of IL-6 and eventually MMP-2, which facilitates tumor migration, invasion, and gliomagenesis [Figure 2C]<sup>[81,82]</sup>. The CX3CR1/IL-1 $\beta$ /CCL2 pathway continues to be an area of active interest, particularly in regard to reduction of IL-6 pro-tumor signaling and inhibition of MMP-based pathways in microglia [Figure 2C and D]<sup>[80]</sup>.

Similarly, studies have examined the p38 MAPK pathway in microglia and its potential for anti-tumor therapy. Minocycline, a tetracycline that inhibits the p38 MAPK pathway, appears to counteract the pro-tumor phenotype of microglia and reduce tumor growth *in vitro* and *in vivo* by inhibiting downstream microglial MT1-MMP expression in mouse models. Decrease in MT1-MMP expression is in turn associated with decreased MMP-2 activity, which follows a similar treatment schema as mentioned above in the IL-1 $\beta$ /CCL2/IL-6 pathway [Figure 2C]<sup>[83,84]</sup>.

Also stimulating secretion of CCL2, IL-6, IL-1, and NO is TNF $\alpha$ , which is readily produced by glioma-associated microglia<sup>[79,85]</sup>. TNF receptor 1 (TNFR1) activation leads to the degradation of I $\kappa$ B $\alpha$ , an inhibitor of NF $\kappa$ B signaling. This degradation leads to a positive feedback loop with p65/p50 nuclear translocation and subsequent transcriptional activation of TNF $\alpha$ <sup>[86,87]</sup>. Meanwhile, NF $\kappa$ B activation also activates pro-migratory genes that contribute to tumor invasiveness involving several pro-tumor chemokines and MMP pathways<sup>[88]</sup>. As such, there is ongoing interest in targeting TNFR1 and the related NF $\kappa$ B pathway in microglia<sup>[86]</sup>.

Moreover, GBM cells induce TLR2/6 activation in both macrophages and microglia *via* the myeloid differentiation primary response 88/TLR8 signaling pathway, which in turn leads to an increase in metalloproteinases like MMP-9 that facilitate tumor invasion and angiogenesis [Figure 2C and D]<sup>[89]</sup>. TLR2 on microglia is also directly involved with promoting tumor invasion with downstream production of MT1-MMP<sup>[90]</sup>. Studies done with murine GL261 glioma cells injected into TLR2 KO mice resulted in smaller tumor burden and reduced MT1-MMP levels in glioma-associated microglia<sup>[90]</sup>.

Contributing further empiric credence to the idea that macrophages and microglia are distinct populations in the TME, Jacobs *et al.*<sup>[91]</sup> found that propentofylline, a methylxanthine, directly acts only on microglia-and not on macrophages-through tumor necrosis factor receptor superfamily, member 19 (TNFRSF19)/TROY inhibition. In a rat glioma model, they demonstrated that TROY is upregulated in infiltrating microglia, with downstream expression of pro-tumor genes Pyk2, Rac1, and pJNK. The potential effectiveness of targeting TROY was shown through a series of siRNA experiments that resulted in inhibition of microglial migration towards glioma cells and, as a result, decreased pro-tumor activity [Figure 2C].

Another important pathway involves signal transducer and activator of transcription 3 (STAT3), which is upregulated in both GBM and glioma-associated microglia and is associated with GBM pathogenesis, progression, and immune evasion<sup>[92-94]</sup>. A study by Lisi *et al.*<sup>[95]</sup> in 2014 examined the use of rapamycin to inhibit mTOR in glioma; the result was the reversal of pro-tumor functions in microglia with glioma-specific cytotoxic behavior. Normally, mTOR leads to an increase in STAT3 expression, which in turn results in increased production of pro-tumor cytokines IL-6, IL-10, CXCL2, and HIF-1 $\alpha$  [Figure 2C-E]. With mTOR

inhibition, iNOS production was increased in glioma-associated microglia along with concomitant decrease in IL-10 gene expression<sup>[94,95]</sup>. Moreover, several preclinical studies have demonstrated that siRNA inhibition of STAT3 in glioma cells leads to microglial activation and tumor growth inhibition in murine models, with increases in IL-2, IL-4, IL-12, IL-15, and CXCL10, along with upregulation of CD80 and CD86 on myeloid cells<sup>[94,96]</sup>.

Finally, the production of HIF-1 $\alpha$ , vascular endothelial growth factor (VEGF), and CXCL2 in myeloid and glioma cells have long since been known to have roles in tumor angiogenesis [Figure 2E]<sup>[97]</sup>. Specific to microglia however is CXCL12 and its ligand CXCR4, which have been implicated in radiation resistance and increased tumor vasculogenesis. Tabouret *et al.*<sup>[98]</sup> demonstrate that with tumor recurrence, there is a switch in expression profile from VEGFR3-HIF-1 $\alpha$  to CXCL12-CXCR4 predominance in glioma-associated microglia. As such, microglia may have roles in propagating additional mechanisms of immune resistance in tumor recurrence, providing another rationale for studying and targeting this population to optimize anti-tumor strategies [Figure 2E].

## CONCLUSION

In this review, we discussed the importance of the roles that microglia and macrophages play in GBM. These two cell types have been shown to be complicit in contributing to an immunosuppressed and/or tumor-progressive milieu; however, more data need to be collected on the interactions between microglia and macrophages within these populations in the TME. This review also highlights the importance of semantically distinguishing between microglia and macrophages. As there are certain cancer-specific interactions with either microglia or macrophages, we recommend clearly delineating between the two in order to avoid complicating future experimental designs and discussions. Furthermore, this review provides a note of caution in strictly following the M1/M2 phenotype for macrophages and microglia, as they are complex and have several differences with each other that make this transposed classification scheme largely unfounded. However, it is this same complexity we appreciate for the potential exploration of new pathways; we look forward to further studying these populations and pathways to work towards a clearer understanding of immunotherapy for GBM.

## DECLARATIONS

### Authors' contributions

Conceived of the presented idea: Choi J, Lim M

Underwent literature review and synthesized a draft: Choi J

Looked over and edited draft: Lim M, Mai N, Jackson C, Belcaid Z

Developed the figures and tables and organized the final draft: Choi J, Mai N

Contributed ideas throughout the process and approved the final draft: Lim M

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All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

**Consent for publication**

Not applicable.

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Review

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# CAR-T cell therapy in neuro-oncology: applications and toxicity

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## Abstract

A new era for cancer treatment has been ushered in with the field of cancer immunotherapy. After initial success with systemic malignancies, several of these promising treatments are being investigated for efficacy with primary and secondary brain tumors. Chimeric antigen receptor (CAR) T cells are being studied, both with systemic infusion and direct administration to the tumor and into the cerebrospinal fluid, with promising early results. Systemic CAR-T treatment can have serious systemic and neurological toxicities that are important for the practicing neurologist and neuro-oncologist to know and understand. This review aims to discuss adoptive cell therapies with a focus on CAR-T treatment. We review use of this therapy in brain cancers, particularly malignant glioma, and provide an overview of the toxicity of CAR-T treatment and its appropriate management.

**Keywords:** CAR-T cell, immunotherapy, brain tumor, cytokine release syndrome

## INTRODUCTION

The immune system plays many roles in cancer prevention. Not only does it protect against viral infections and the development of virus-driven tumors, it also eliminates tumor cells, and rapidly identifies and



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eliminates foreign pathogens, minimizing the duration of an inflammatory, tumor-promoting environment. It is also understood that the immune system can impact the immunogenicity of the tumor itself given intact immune systems impact the very nature of the tumor and contribute to its ability to progress and grow. Understanding the impact of the immune system, including this knowledge of “cancer immunoediting” has allowed for the development of additional therapies to treat cancer<sup>[1]</sup>. Various classes of immunotherapies now exist that attempt to harness and exploit different strengths of the immune system, ranging from adoptive T cell therapy and cytokine therapy to checkpoint blockade therapy and oncolytic virus therapy<sup>[2]</sup>. Our improved understanding of the central nervous system (CNS) over the last several decades has led to the growth and continuous innovative design of immunotherapeutics for different malignancies, including those in the CNS. Lymphatics have been identified in the meninges and the dura, demonstrating a communication route for lymphocytes to and from the brain and cervical lymph nodes<sup>[3]</sup>. We also now know that the CNS has processes of immunosurveillance which allow for autoimmune disease and paraneoplastic syndromes to exist within its realm. The immune system can thus be used to our advantage in fighting cancer even in the CNS, contrary to prior belief. It has been shown that T cells infused intravenously will make their way to the central nervous system, but there are still many challenges to resolve before we are able to harness the immune system to attack cancer without excessive toxicity<sup>[4]</sup>. The responses to antigens must be narrow and limited only to those particular epitopes; otherwise, the response can spread and damage healthy, normal brain tissue. Tumor specific mutations are also less common in brain tumors compared to other malignancies, and may be unique to each individual, requiring optimal individualized medicine which can be cost-prohibitive<sup>[5]</sup>. Another challenge is that gliomas appear to have natural mechanisms to inhibit T cell activation and actually decrease peripheral T-cell counts, resulting in the lymphopenia seen in these patients even prior to the initiation of therapy<sup>[6]</sup>. Glioma cells are even able to evade detection by the immune system by down-regulating major histocompatibility complexes (MHC) or other components necessary for antigen presentation<sup>[7]</sup>. Thus, there are many avenues and opportunities for further exploration in the field of immunotherapy as it relates to brain tumor treatments, and as we learn and understand more, we slowly improve in our ability to develop novel therapeutics for CNS tumors.

## ADOPTIVE CELL THERAPIES - INTRODUCTION TO CHIMERIC ANTIGEN RECEPTOR-T

The ability to harness the body's own immune system to create a sustained anti-tumor response has transformed conventional therapeutic strategies for patients with cancer. Cell-based therapies involve the allogeneic or autologous transfer of immune-derived cells into cancer patients to enhance the host immune system's ability to recognize and destroy cancer cells<sup>[8,9]</sup>. T-lymphocytes are the backbone of the body's defense system due to their critical role in orchestrating and executing an effective immune response. They have been identified as prime candidates for their robust response against foreign pathogens and persistent anti-tumor activity<sup>[10]</sup>. Although endogenous T cells are frequently unable to eradicate progressing tumor on their own, modern technology has made it possible to genetically engineer the function, specificity, and longevity of T-cell anti-tumor response. Perhaps most importantly, the adoptive transfer of these cells can elicit a high degree of efficacy and specificity, thus, minimizing off-target toxicities.

Adoptive T-cell therapy (ACT) consists of isolating, expanding and reintroducing tumor-specific T lymphocytes into patients with cancer. Currently, there are four forms of ACT actively being investigated for cancer treatment: (1) tumor-infiltrating lymphocytes (TILs); (2) cytotoxic T lymphocytes (CTLs); (3) T-cell receptors (TCRs); and (4) chimeric antigen receptors (CARs) [Table 1]<sup>[9,11]</sup>. TILs have unique anti-tumor properties as they are extracted from tumor biopsies where they have been exposed and conditioned to the tumor microenvironment. These cells can be isolated from tumor tissue and expanded in IL-2 cytokine prior to re-introduction and have shown to be an effective approach for patients with metastatic malignant melanoma<sup>[12]</sup>. CTLs are naturally circulating tumor-specific T cells taken from a patient's peripheral blood and can be expanded using antigen presenting cells (APCs). Both TILs and CTLs, once infused back into the patient, recognize tumor associated antigens (TAA) via their TCR as antigenic peptides presented by MHC,

**Table 1. Types of adoptive T cell therapies. Summary of T cell therapeutic strategies, mechanistic action, benefits and limitations**

Modified T-cell product	Description	Mechanism	Benefits	Limitations
TILs	Natural T lymphocytes extracted from autologous tumor biopsy and ex vivo cytokine fortification	Recognition of TAA via MHC-I complex by conditioned TCRs	Extensive intracellular and extracellular TAA specificity and recognition	Restricted to MHC-I complex Isolation and expansion complexities Low frequency of antigenic peptides expressed by specialized cells
CTLs	Enhanced and expanded in ex vivo isolated circulating tumor-specific T cells extracted from the patients' peripheral blood using APCs	Via MHC-I complex, enriched TCRs recognize processed TAAs	Highly advantageous of helper T cells and APC augmentation of persistent anti-tumor activity and prolonged survival <i>in vivo</i> . Specific to viral or non-viral antigenic peptides	Restricted to MHC-I complex. Limited efficacy in non-viral tumor-specific antigenic peptides Low affinity of TAA TCR. Low frequency of TAA specific cells
TCRs	T lymphocytes modified to express a tgTCR with optimal specificity towards TAAs	Recognition of tumor antigenic peptides by tgTCR presented on MHC-I molecules	Augmented specificity in targeting extracellular or intracellular TAAs	Restricted to MHC-I complex and HLA-A2 patients tgTCR genetic mis-match with native TCR
CARs	Engineered T cells expressing an antibody-binding scFv exodomain fused with a CD3z chain intracellular domain via a transmembrane domain	CAR extracellular domain recognizes and binds to specific TAAs in a MHC-independent manner	Highly specific modified T cells with reliable production and unrestricted to any MHC complexes	Cytokine release syndrome largely due to persistent T cell proliferation and subsequent cytokine secretion

TILs: tumor infiltrating lymphocytes; CTLs: cytotoxic T lymphocytes; TCR: T cell receptor; CARs: chimeric antigen receptors; IL-2: interleukin-2; TAA: tumor-associated antigens; MHC-I: major histocompatibility complex I; APCs: antigen presenting cells; tgTCR: transgenic TCR; HLA: human leukocyte antigen; scFv: single-chain variable fragment

and subsequently execute T cell activation<sup>[13]</sup>. T cells can also be engineered to express TCRs and the ability to genetically clone, and affinity optimize TCRs can substantially increase their potential in recognizing tumor-specific antigens<sup>[14,15]</sup>. Lastly, CARs are genetically engineered surface receptors composed of extracellular antigen-binding domains fused to intracellular T cell signaling domains of the T-cell receptor. These modified T-cells expressing tumor-targeted CARs redirect antineoplastic specificity towards cancer cells without MHC restriction. CARs have advanced the furthest in clinical development with their recent FDA approval and their use showing remarkable clinical outcomes for patients with hematological malignancies<sup>[14-19]</sup>.

CARs consist of an extracellular recognition domain that can bind specifically to a target molecule expressed on the surface of tumor cells, and an intracellular signaling domain that provides an activation signal upon target binding, linked via a transmembrane spacer/hinge domain. The extracellular domain is usually comprised of an antibody single-chain variable fragment (scFv)<sup>[20,21]</sup>. Interestingly, ligands of cell-surface receptors are also alternative molecules being used at multiple institutions<sup>[22,23]</sup>. The transmembrane spacer domain is important in conferring stability, flexibility and spacial orientation for CAR-antigen immunological synapse formation. Once the extracellular domain binds to a tumor-specific antigen, it will communicate and activate the intracellular domain, which directly initiates CAR-T cell cytolytic activity, cytokine production and proliferation<sup>[24]</sup>. The intracellular domain usually incorporates a region of the TCR CD3z chain to provide the primary activating signal. Most CAR designs also incorporate one or more domains from co-stimulatory receptors, such as CD28, CD134 (OX40), and/or CD137 (4-1BB) to provide the secondary signal for the optimal activation of downstream signaling cascades<sup>[25,26]</sup>.

Over the past two decades, CAR-T cell designs have dramatically improved in their ability to mediate anti-tumor activity. The first-generation CARs only included a CD3z signaling domain, and although these first-generation CARs were sufficient to redirect T cell cytotoxic activity, they exhibited suboptimal persistence and *in vivo* killing potential. By contrast, second generation CARs include the addition of a costimulatory signaling domain (i.e., CD28, CD134, CD137) to enhance the signal function of the CD3z

signaling domain. Indeed, second generation CARs have shown robust antitumor activity in patients in the setting of hematological malignancies. In third generation CARs, the main difference compared to second-generation CARs is the inclusion of two costimulatory signaling domains. The costimulatory additions aim to amplify the anti-tumor effect of second generation CARs, however, it remains to be determined whether the inclusion of additional co-stimulatory domains improves CAR function. The fourth-generation CARs are referred as T-cell redirected for universal cytokine killing (TRUCK) - and a variety of cytokine genes have been added to this structure<sup>[13]</sup>. There have also been recent creative designs that involve infusion of nanoparticles aimed to enhance T-cells anti-tumor response<sup>[27]</sup> and synthetic Notch (synNotch) receptors to increase tumor specificity<sup>[28]</sup>. Inducible CAR systems are also being developed by many researchers centered around the idea that the T cells would only turn “on” and become cytotoxic in the presence of certain other drugs - for example, a tetracycline regulation system that only turns on with doxycycline introduction<sup>[12]</sup>. Dual targeting CARs have also been developed that can target different receptors and induce signaling that will results in different outcomes - for example, one pathway elicits cytotoxicity while another promotes the proliferation of T cells<sup>[14]</sup>.

### CAR-T CELLS IN SYSTEMIC CANCER

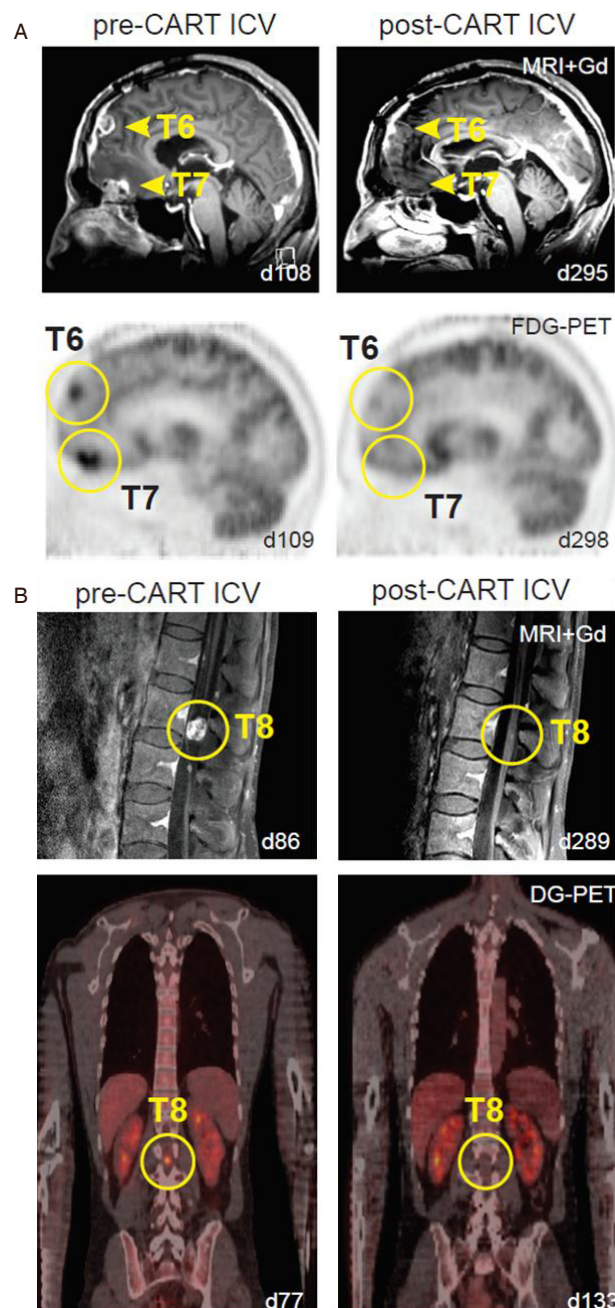
The first clinical application of CD19 CAR-T therapy allowing for the products to be studied for their efficacy in humans was initiated in 2005 (NCT00182650; PMID: 20304086). Multiple clinical trials since then have shown impressive outcomes in patients with relapsed, refractory B-cell hematologic malignancies. This includes pediatric lymphoblastic leukemia (ALL), aggressive B cell non-Hodgkin's lymphoma, and chronic lymphocytic leukemia (CLL)<sup>[15-21]</sup>. The response has not only been dramatic in terms of overall response rate and complete remission rate progression-free survival, but it has also been very durable in the majority of cases. Consequently, on August 30th, 2017, the first anti-CD19 CAR-T cell product, tisagenlecleucel, was approved for the treatment of children and young adults with relapsed or refractory B-cell precursor ALL and subsequently also for the treatment of aggressive B cell lymphoma<sup>[21,22]</sup>. This was followed by the approval of axicabtagene ciloleucel, another autologous anti-CD19 CAR-T cell therapy for the treatment of relapsed large B-cell lymphoma, in October of 2017<sup>[23]</sup>.

### CAR-T CELLS IN BRAIN CANCER

CAR-T cell therapy has shown tremendous clinical success in the treatment of hematological malignancies and also solid tumors extending to the central nervous system (CNS). Within the JCAR017 clinical trial, Abramson reported a case study of a 68-year-old patient with a systemic refractory diffuse large-B-cell lymphoma who had developed a new brain lesion in the right temporal lobe. The patient was given CD19-targeted CAR-T cells and exhibited complete remission for 12 months in the absence of any neurotoxicity<sup>[24]</sup>. Interestingly, CAR-T cells were identified in the cerebrospinal fluid (CSF) after systemic administration, suggesting an alternative delivery route and their ability to cross the blood brain barrier, and trigger a durable anti-tumor response<sup>[25,26]</sup>.

Several clinical trials across the nation are evaluating the efficacy of CAR-T cell therapy for patients with primary neuro-oncological malignancies. Multiple institutions are looking into several tumor targets as well as alternative delivery routes for optimal anti-tumor response. Brown *et al.*<sup>[27]</sup> described three patients with recurrent glioblastoma treated with first-generation interleukin-13 receptor alpha 2 (IL13Rα2) redirected CAR-T cells. Using an Ommaya reservoir, patients were given up to 12 intracavity infusions with two patients showing transient antitumor response. CNS inflammation was observed in all three patients and the degree of inflammation appeared to correlate with IL13Rα2 antigen expression. This indicated a persistent anti-tumor response through the activation of CAR-T cells once they recognized and were bound to the TAA<sup>[27]</sup>. Additionally, Brown *et al.*<sup>[28]</sup> reported the use of second-generation CAR-T cells targeting IL13Rα2 in a patient with recurrent multifocal leptomeningeal glioblastoma (GBM). The patient showed no disease progression at the tumor site receiving CAR-T cell infusions via an Ommaya catheter; however this intratumoral





**Figure 1.** Multifocal glioblastoma responding to intraventricular delivery of IL13Ra2-redirected CAR T-cell therapy (modified from Brown *et al.*<sup>[28]</sup>, permission for use granted by NEJM). A: sagittal view of gadolinium-enhanced MRI (top row) and FDG-PET (bottom row) images showing tumor regression of tumors 6 and 7 in the brain (yellow arrowheads and circles); B: sagittal gadolinium-enhanced MRI (top row) and coronal DG-PET (bottom row) images exhibiting tumor regression of tumor 8 in the spine (yellow circles)

treatment failed to prevent the development of additional tumors over time. Due to metastatic progression, the patient subsequently received 10 intrathecal infusions into the right lateral ventricle. This was the first time intraventricular administration of CAR-T cells was performed and surprisingly showed remarkable tumor regression at the 6th infusion (out of 10). Complete response was observed for 7.5 months with increased production of cytokines and immune cells but without any major systemic associated side effects. The study suggested the ability to induce transient anti-tumor activity against multifocal glioblastoma with leptomeningeal seeding through multiple delivery routes with very manageable therapy-related toxicity [Figure 1]<sup>[28]</sup>. O'Rourke *et al.*<sup>[29]</sup> reported their experience with 10 patients with recurrent GBM who received a single dose

**Table 2. List of clinical trials investigating the efficacy of chimeric antigen receptor T-cell therapy across multiple antigens expressed in brain cancer**

Antigen	Disease	Intervention/treatment	Identifier
MUC1	Malignant glioma	Anti-MUC1 CAR-T cells in patients with malignant glioma	NCT02617134
EGFRvIII, IL12Ra2, HER2, CD133, EphA2, GD2	Recurrent malignant glioma	CAR T cells redirected to target tumor specific/associated antigens in patients with recurrent malignant gliomas	NCT03423992
HER2	Pediatric CNS tumors	HER2-specific CAR T cell for recurrent/refractory pediatric CNS tumors	NCT03500991
EGFRvIII	Malignant gliomas	Anti-EGFRvIII CAR T cells in patients with malignant glioma	NCT01454596
IL13Ra2	Recurrent malignant glioma	IL13Ra2-specific, hinge-optimized, 41BB-costimulatory CAR/truncated CD19-expressing autologous T lymphocytes	NCT02208362
CD133	Glioma	Anti-CD133 modified CAR T cells in relapsed patients with malignant glioma	NCT02541370
HER2	GBM	CMV-specific CTLs expressing CAR targeting HER2 positive recurrent GBM patients	NCT01109095

CAR: chimeric antigen receptor; CNS: central nervous system; CTLs: cytotoxic T lymphocytes



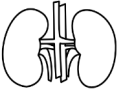






of peripherally infused EGFRvIII directed CAR-Ts. In their phase-I open label study, all 10 patients had an unmethylated MGMT promoter, known to be a poor prognostic factor. The median level of EGFRvIII expression was 71% (6%-96%). Importantly, there were no dose-limiting toxicities, and CAR-T therapy was not associated with EGFR-directed toxicity, systemic toxicity (CRS - see below) or neurotoxicity - signs and symptoms observed with CD-19- CAR-T cells. Median overall survival (OS) was 251 days (8 months) with one subject alive without further therapy 18 months after a single infusion of CART-EGFRvIII<sup>[29]</sup>.

Other tumor biomarkers expressed by GBM are being tested in several institutions across the world. Yang and colleagues at Hefei Binhu Hospital are testing a modified CAR-T cell redirected to target MUC1 positive tumors in patients with recurrent/relapsed malignant glioma (NCT02617134). Lin and colleagues at Xuanwu Hospital in China are recruiting patients with recurrent malignant gliomas to test a spectrum of tumor specific/associated antigens including EGFRvIII, IL12Ra2, HER2, CD133, EphA2 and GD2 (NCT03423992). Rosenberg's group from the National Cancer Institute is investigating an anti-EGFRvIII CAR in patients with malignant glioma who are positive for EGFRvIII (NCT01454596). In a phase I clinical trial, Vitanza from Seattle Children's Hospital is testing a HER2-specific CAR in recurrent/refractory pediatric patients diagnosed with CNS tumors (NCT03500991). At Baylor College of Medicine, Ahmed and colleagues developed an autologous second-generation Cytomegalovirus (CMV)-specific CAR targeting HER2-positive GBM cells. It is currently undergoing evaluation in a clinical trial for patients diagnosed with GBM (NCT01109095). Chinese PLA General Hospital has deployed a phase I clinical trial investigating an anti-CD133 CAR in relapsed patients with advanced malignancies including brain tumors (NCT02541370). Brown *et al.*<sup>[28]</sup> at the City of Hope Medical Center have developed and deployed a phase I clinical trial testing a second-generation IL13Ra2-specific CAR in recurrent patients with malignant glioma (NCT02208362). Her group is also testing single and combined delivery routes including intracavity, intratumoral and intraventricular in order to identify the optimal route for T-cell infusion that will maximize antitumor response. These studies are further summarized in Table 2.

## SYSTEMIC AND NEUROLOGICAL TOXICITY OF CAR-T CELLS

CAR-T treatment is not without toxicities, which have been well-studied over the years for all of the different products created and investigated in clinical trials. One of these products (JCAR015) had to be taken off the market after the death of five patients from severe cerebral edema and herniation<sup>[30]</sup>.

The primary toxicity that has been seen across trials and is now well-described is cytokine release syndrome or cytokine release syndrome (CRS). This is characterized by fever, malaise, anorexia, myalgias, hypotension, and can include multi-organ dysfunction. The mechanism continues to be explored but the release of

Organ System	Symptoms	
 <b>Systemic</b>	<ul style="list-style-type: none"> <li>Fever</li> <li>Malaise</li> <li>Anorexia</li> <li>Myalgias</li> </ul>	
 <b>Neurological</b>	<ul style="list-style-type: none"> <li>Headaches</li> <li>Altered mental status</li> <li>Delirium</li> <li>Hallucinations</li> <li>Aphasia</li> <li>Apraxia</li> </ul>	<ul style="list-style-type: none"> <li>Ataxia</li> <li>Dysmetria</li> <li>Tremor</li> <li>Myoclonus</li> <li>Seizures</li> </ul>
 <b>Renal</b>	<ul style="list-style-type: none"> <li>Acute kidney injury</li> <li>Hyponatremia</li> <li>Hypokalemia</li> </ul>	<ul style="list-style-type: none"> <li>Hypophosphatemia</li> <li>Tumor lysis syndrome</li> </ul>
 <b>Hepato-biliary</b>	<ul style="list-style-type: none"> <li>Transaminitis</li> <li>Hyperbilirubinemia</li> </ul>	
 <b>Gastrointestinal</b>	<ul style="list-style-type: none"> <li>Nausea</li> <li>Emesis</li> <li>Diarrhea</li> </ul>	
 <b>Cardiovascular</b>	<ul style="list-style-type: none"> <li>Tachycardia</li> <li>Hypotension</li> <li>Arrhythmias</li> </ul>	<ul style="list-style-type: none"> <li>Decreased left ventricular ejection fraction</li> <li>Troponinemia</li> <li>QTprolongation</li> </ul>
 <b>Musculoskeletal</b>	<ul style="list-style-type: none"> <li>Myalgias</li> <li>Weakness</li> <li>Creatine kinase elevation</li> </ul>	
 <b>Pulmonary</b>	<ul style="list-style-type: none"> <li>Tachypnea</li> <li>Hypoxia</li> </ul>	
 <b>Hematologic</b>	<ul style="list-style-type: none"> <li>Anemia</li> <li>Thrombocytopenia</li> <li>Neutropenia</li> <li>Lymphopenia</li> <li>B cell aplasia</li> <li>Prolonged prothrombin time</li> </ul>	<ul style="list-style-type: none"> <li>Hypofibrinogenemia</li> <li>Prolonged activated partial thromboplastin time</li> <li>Elevated D-dimer</li> <li>Disseminated intravascular coagulation</li> <li>Hemophagocytic lymphohistiocytosis</li> </ul>

**Figure 2.** Toxicity from chimeric antigen receptor-T by organ system. Chimeric antigen receptor-T cell treatment has been found to result in a number of toxicities throughout the body. Professional illustration by Ryan Stemen

**Table 3. Cytokine release syndrome and chimeric antigen receptor-T cell related encephalopathy syndrome\***

Grade	CRS	CRES
Grade 1	Non-life threatening symptoms (includes fever, nausea, fatigue, headache, myalgias, grade 1 organ toxicity)	CARTOX-10 score 7-9 (mild impairment)
Grade 2	Symptoms require moderate intervention (Oxygen requirement < 40%, hypotensive but responsive to fluids or low dose pressors, grade 2 organ toxicity)	CARTOX-10 score 3-6 (moderate impairment)
Grade 3	Symptoms require aggressive intervention (Oxygen requirement ≥ 40%, hypotensive requiring high dose or multiple pressors, grade 2 organ toxicity or grade 4 transaminitis)	CARTOX-10 score 0-1 (severe impairment), intracranial pressure as noted by papilledema or CSF opening pressure < 20 mmHg, partial seizures or non-convulsive seizures on EEG responsive to benzodiazepines.
Grade 4	Life-threatening symptoms (ventilator support, grade 4 organ toxicity)	Obtunded, high grade papilledema or CSF opening pressure ≥ 20 mmHg or cerebral edema on imaging, generalized seizures or status epilepticus, new motor weakness
Grade 5	Death	

\*Adapted from Neelapu *et al.*<sup>[23]</sup> and Lee *et al.*<sup>[32]</sup>. CAR: chimeric antigen receptor; CRS: cytokine release syndrome; CRES: CAR-T related encephalopathy syndrome; CSF: cerebrospinal fluid

cytokines by infused T-cells plays a significant role in this syndrome. A variety of inflammatory cytokines have been found to be elevated in the serum of patients experiencing CRS, including interleukin(IL)-6, interferon-gamma, IL-15, IL-8, IL-10, and IL-2. Higher disease burden has been predicted to lead to more toxicity, but other factors continue to be explored. Various organ systems have been noted to be impacted by CRS to varying degrees<sup>[23,31]</sup> [Figure 2]. CRS is generally seen within the first week after infusion of CAR-T cell therapy and the peak risk is within the first two weeks of administration - thus, patients are typically monitored in the acute inpatient setting (with access to an intensive care unit), and with frequent monitoring of vital signs and laboratory parameters including blood counts, coagulation factors, measures of organ function, and inflammatory markers<sup>[23,31]</sup>.

Various grading systems to evaluate CRS have been developed including one by Lee *et al.*<sup>[32]</sup> which has been used at various centers where the therapy is offered<sup>[32]</sup>. The group modified a National Cancer Institute Common Terminology Criteria for Adverse Events or CTCAE to define mild, moderate, severe and life-threatening degrees of CRS. Grade 1 symptoms are mild, requiring supportive treatment, and include fever, nausea, headache, *etc.* The grading is subsequently raised based on the degree of organ toxicity noted and the level of intervention required to maintain a stable hemodynamic status, with grade 5 toxicity translating to death [Table 3]<sup>[32]</sup>.

Neurological toxicity is the other well-described and important phenomenon seen in these patients and has been noted from the very onset of CAR-T trials. A wide spectrum of symptoms have been reported including headaches, global encephalopathy, seizures, tremors, ataxia, hemiparesis, aphasia, ataxia, apraxia, dysmetria, and cranial nerve palsies. In very rare cases, diffuse cerebral edema has been noted, which has in some cases been fatal. Elevated cytokine levels are implicated although it is not yet completely clear what is the mechanism of neurological dysfunction observed across patients. The role of endothelial activation and increased blood-brain barrier permeability as well as role of IL-1 have been recently described in association with neurotoxicity<sup>[33]</sup>. Evaluation of several cases has demonstrated anti-CD19 CAR T cells may be found at a higher level in the CSF of patients who experience neurotoxicity, and these patients may also have higher levels of IL-6 in the CSF<sup>[32,34]</sup>. While the initial neurotoxicity is seen early in the course with concurrent CRS, delayed neurological toxicity up to several weeks after infusion has also been noted, though the mechanism for this remains unclear.

Neelapu *et al.*<sup>[23]</sup> have described the neurological toxicity seen with CAR-T infusions and developed the term CAR-T related encephalopathy syndrome (CRES), and have provided a helpful grading system for this entity<sup>[23]</sup>. Similar to CRS, Grade 1-4 are assigned based on a combination of a neurological assessment

score, raised intracranial pressure and seizures or motor weakness. The neurological status is assessed with CARTOX-10, a 10-point neurological assessment tool developed by the authors that evaluates attention, speech and writing ability [Table 3].

It should be noted that direct CNS infusions of CAR-T have not resulted in the same toxicity (CRS or CRES), though only a few patients have had this treatment at this point. As previously discussed, Brown *et al.*<sup>[28]</sup> described a patient with glioblastoma who was treated with an intrathecal delivery of CAR-T cells. They reported only grade 1-2 toxicity, the majority of which lasted less than 1 day. Grade 1 fatigue was the most lasting adverse event (4 days). Headache, myalgias and lymphopenia were the worst higher grade toxicities (Grade 2)<sup>[28]</sup>.

## MANAGEMENT OF CAR-T TOXICITY

Management of CAR-T associated toxicity is based on severity of symptoms. It begins with symptomatic management and supportive care for milder symptoms - fever is treated with acetaminophen or cooling blankets, acetaminophen is used for myalgias, anti-emetics for nausea, *etc.* Intravenous (IV) fluids may be started for hydration in grade 1 toxicity. Organ systems are closely monitored with frequent laboratory assessments and organ toxicities treated according to what is seen (for example, acute kidney injury or AKI may be treated with hydration). Higher grades of CRS require greater intervention. Oxygen requirements may need to be met with supplemental oxygen and/or even ventilation. Vasopressors may need to be started and titrated to maintain a stable hemodynamic status. Ultrasound may be needed to assess hemodynamic status, and frequency of vitals and assessments may need to be increased. For these reasons, patients who are not yet in the intensive care unit (ICU) are often transferred when CRS or neurotoxicity is grade 2 or higher<sup>[23,31,32,35]</sup>.

Similar to CRS, management of CRES is also escalated depending on the grade of the toxicity seen. Careful and frequent neurological assessment by experienced providers is extremely important. In addition, it is also important to use a scale such as the CARTOX-10 or a similarly sensitive tool that can pick up on subtle, early neurological deficits. With even minimal signs of neurological impairment, the patient is generally placed on aspiration precautions. The patient must be closely and frequently monitored from this point onwards with careful neurological examinations by a neurologist, and regular funduscopic exams. CT head of the brain, followed by MRI of the brain (and/or spine if appropriate and if deficits are present) is usually obtained along with a lumbar puncture to assess opening pressure and rule out other conditions that these patients may be at risk for (such as leukemic or infectious meningitis). If the patient is too unstable, a CT of the brain can be obtained instead of MRI in this early stage. If patient is noted to be encephalopathic, an EEG should be considered to rule out non-convulsive status epilepticus<sup>[23]</sup>.

If the patient does develop increased intracranial pressure or has seizures (convulsive or non-convulsive), management should be guided per the protocols for each of these conditions. An experienced neurology team is thus quite necessary. Increased intracranial pressure may be managed with dexamethasone, mannitol or acetazolamide, bed elevation, hyperventilation, or hyperosmolar therapy, or even drainage of CSF. Seizures, and possible status epilepticus, should be managed per nationally established guidelines that include lorazepam as a starting point with IV boluses of anti-epileptics such as levetiracetam and escalating to additional medications or a drip of midazolam or phenobarbital if seizures continue despite early efforts. Drugs such as fosphenytoin and lacosamide are typically avoided due to their potential for additional cardiotoxicity. Some centers are using prophylactic levetiracetam (at 500 mg BID) prior to starting the treatment process, though at this time no clear evidence supports the use of prophylactic anti-epileptics<sup>[23]</sup>.

Tocilizumab is an IL-6 antagonistic monoclonal antibody that has been available on the market to treat rheumatological disorders. Given the high levels of IL-6 seen with the toxicity of CAR-T, it was tried and



found to effectively treat CRS-related complications in clinical trials. Eventually, it was approved by the FDA for this specific purpose in 2017. It is recommended as the first course of treatment when CRS and neurological toxicity is confirmed as per the grading criteria discussed previously. Tocilizumab can be dosed at 8mg/kg IV for CRS or CRES, starting as early as grade 1 toxicity. Siltuximab, an anti-IL-6 chimeric monoclonal antibody, can also be used instead of tocilizumab in these cases, and is dosed at 11mg/kg IV. If the patient fails to respond to these drugs and proceeds to grade 2 or higher CRS/CRES, then corticosteroids may need to be used. Corticosteroids appear to be more effective for patients with neurotoxicity only without concomitant CRS. Dexamethasone is typically dosed at 10mg IV every 6 h, and continued toxicity without response to dexamethasone may need to be treated with methylprednisolone. There has been concern that steroids may impact the inflammatory response that is important for the efficacy of CAR-T cell treatment and may suppress the function of the tumor-directed T cell therapy since they can suppress T cells and induce apoptosis of these cells. However, preliminary data at this point demonstrates no clear objective difference in response rates<sup>[36]</sup>. Most management protocols advise considering steroids if patients are demonstrating grade 2 toxicity without response to tocilizumab or siltuximab<sup>[23,31]</sup>.

## CONCLUSION

CAR-T therapy has had promising results in hematological malignancies and has revolutionized the immunotherapy field in the last several years. There is a great hope that with the identification of the right antigens, and with improvements in delivery and safety, CNS malignancies might be successfully treated with this therapy. Early studies are demonstrating some encouraging results, but larger trials are needed to fully evaluate this modality in treatment of brain cancer (both primary and secondary). It is critically important for the providers to understand the principles of this therapy, its mechanism of action and particularly its neurologic toxicity. While the understanding of neurotoxicity continues to evolve, early integration of the neurology team in the care of patients receiving CAR-T cell therapy clearly provides benefit. Immunotherapy is an emerging area in oncology and its use, including the indications for CAR-T, are likely to expand.

## DECLARATIONS

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### Authors' contributions

Researched literature, wrote the manuscript: Sharma A, De Leon G

Provided content, reviewed the manuscript: Porter A, Grill MF, Rosenthal A, Swanson K

Provided content and figures, reviewed the manuscript: Brown CE

Article concept, literature research, writing and reviewing the manuscript: Mrugala MM

### Availability of data and materials

Data available on PubMed and [www.clinicaltrials.gov](http://www.clinicaltrials.gov).

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Not applicable.

### Consent for publication

Not applicable.

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Correction

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## Correction: *In silico* design of novel gold-phosphate containing compounds as selective inhibitors of cathepsin B in neuroinflammation

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Dr. Mohsen Sharifi declares the view presented in the article are those of the authors and do not reflect those of the US Food and Drug Administration. No official endorsement is intended nor should be inferred.



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Review

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# Total tumor RNA pulsed dendritic cells plus adoptive transfer of *ex-vivo* enriched autologous T-lymphocytes in the treatment of children with primary brain tumors

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## Abstract

The therapeutic approach of adoptive lymphocyte transfer (ALT) using lymphocytes primed and expanded *ex-vivo* by exposure to total tumor RNA (ttRNA) containing dendritic cells (DCs) and administered after lymphodepletive host conditioning in patients with refractory melanoma with brain metastases has shown excellent objective responses indicating that the central nervous system (CNS) is not an immune privileged site and further paved the way for utilization of a similar approach in other cancers. We have shown that the use of ALT + ttRNA DCs following either myeloablative or non-myeloablative host conditioning is feasible and safe and appears to prolong survival in a proportion of children with recurrent medulloblastoma who had failed standard cytotoxic therapy. Further refinements in this promising approach are needed to improve outcomes and extend this treatment to a broad range of CNS malignancies.

**Keywords:** Children, brain tumors, immunotherapy, adoptive lymphocyte transfer, dendritic cell vaccine, lymphodepletion, total tumor RNA

## INTRODUCTION

### Primary pediatric central nervous system tumors

Pediatric brain tumors are the commonest solid tumors in children and unfortunately the cause for the most cancer related mortality in this age group<sup>[1]</sup>. Standard treatment for these patients at diagnosis includes



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surgery, chemotherapy, and/or irradiation leading to cures around 70% overall but with a high cost in terms of permanent neuro-cognitive and hormonal deficits<sup>[2]</sup>. The prognosis for those with recurrent disease remains dismal and novel approaches are urgently needed for refractory tumors. With the recent spurt in enthusiasm in the field of cancer immunotherapy, there is an increasing interest to utilizing this approach to treat children with recurrent central nervous system (CNS) tumors. The focus of this review is our experience in using adoptive lymphocyte transfer (ALT) with total tumor derived mRNA loaded dendritic cells (DCs) following myeloablative (MA) conditioning chemotherapy in pediatric brain tumors. This work is a form of adoptive cellular therapy (ACT) pioneered in large part by Dr. Rosenberg at the National Cancer Institute that resulted in durable remissions in patients with metastatic melanoma<sup>[3]</sup> and heralded a new approach in the field of adoptive cellular immunotherapy that could be extended to other cancers.

### **The CNS is not an immune privileged site**

The CNS and by extension brain tumors have been thought to be protected from the immune system<sup>[4,5]</sup>. Several lines of evidence lent credence to this theory including an intact blood brain barrier (BBB), lack of prototypical lymphatic structures, a general lack of antigen presenting cells (APCs) within the brain tissue, low to absent expression of major histocompatibility (MHC) class I molecules, constitutive expression of immunosuppressive cytokines including transforming growth factor- $\beta$  and IL-10, and the slow rejection allogeneic tissue implanted in the brain as compared to other sites<sup>[5]</sup>. However, there is also ample evidence to the contrary including the occurrence of paraneoplastic syndromes in which a spontaneous immune response to tumors causes immune damage to the central and peripheral nervous system structures<sup>[6]</sup>, objective immune responses in brain metastases in patients with recurrent metastatic melanomas treated with adoptive T-cell therapy<sup>[3]</sup>, and immune infiltrates seen in primary CNS tumors including malignant gliomas that are a few examples of how the CNS cannot be considered an immune privileged site<sup>[5]</sup>. While the BBB is relatively impermeable in the normal state, tumors generally disrupt the BBB, and tumor release of inflammatory cytokines can further induce migration of immune cells into the brain<sup>[5]</sup>. Released tumor antigens can either be engulfed by APCs and migrate through the cerebrospinal fluid and exit from the cribriform plate to the nasal mucosa or be transported via the interstitial fluid and drain along the capillary walls of blood vessels to reach cervical nodes to sensitize the immune system. Microglia (the CNS resident macrophages) can also play a role through innate or adaptive immunity mechanisms<sup>[5]</sup>.

### **IMMUNE SYSTEM AND CANCER**

The immune system and the responses thereof have been traditionally divided into innate and adaptive immunity with a considerable interaction and cross talk between the two systems<sup>[7]</sup>. Innate immunity is mediated by phagocytes (DCs, macrophages, neutrophils), natural killer (NK) cells, mast cells, eosinophils, and basophils<sup>[8]</sup>. Innate immunity is mediated through receptors that are pre-determined in the germline by approximately 100 genes and are called pattern recognition receptors targeting a specific set of ligands grouped as pathogen or damage associated molecular patterns<sup>[8]</sup>. Innate responses are rapid and occur within a matter of hours following a pathogenic threat.

The key players in the acquired form of immunity include the APCs, T-helper (CD4), T-suppressor cells, and cytotoxic CD8+ cells<sup>[9]</sup>. Acquired immune responses depend on T and B cell receptor diversity generated somatically during lymphocyte development that are not genetically pre-determined towards a specific antigen and arise on exposure to a pathogen or foreign antigen by random site specific recombination in the immunoglobulin (B-cells) or T-cell receptor (TCR) genes and clonal expansion of such lymphocytes on further antigen exposure<sup>[7,9]</sup>. Degeneracy (in contrast to specificity) is a typical characteristic of antigen recognition in adaptive immunity, which refers to the cellular response from a single receptor that interacts with several ligands (antigens in this case) that are structurally different<sup>[7]</sup>. Acquired immune responses on an average takes 7-10 days to initiate and peak following antigen encounter.

## APCs

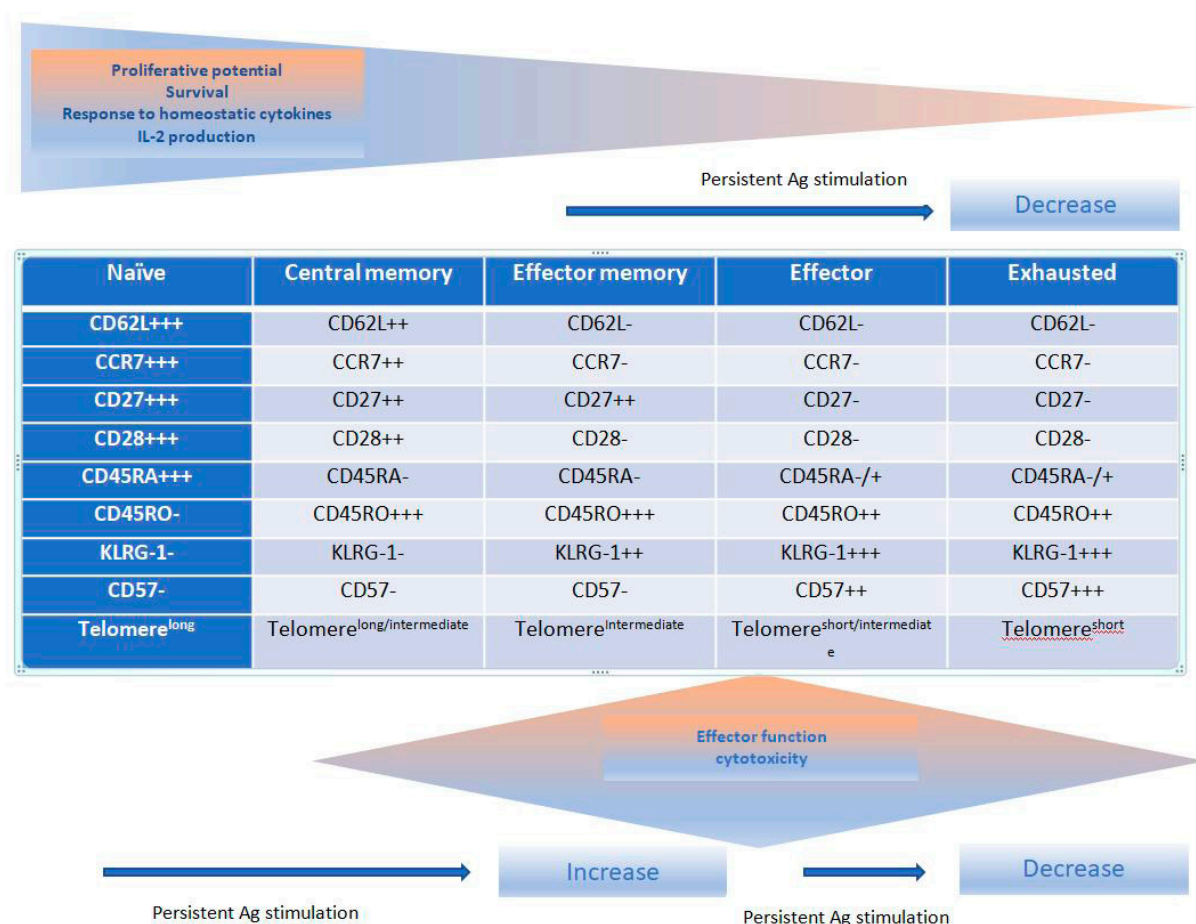
Amongst several APCs, DCs are the key APC in humans<sup>[10-12]</sup>. The human DC arises from a common bone marrow CD14<sup>+</sup> or CD14<sup>-</sup> myeloid progenitor that also gives rise to the monocyte and is uniquely dependent on Flt3-ligand for proliferation<sup>[13,14]</sup>. Antigen presenting cells typically present endogenous or exogenous antigens on the cell surface in the context of MHC complex class I (for endogenous proteins) or class II (for exogenous proteins) molecules<sup>[12]</sup>. The proteasomes within each cell converts proteins into small peptide fragments (15-20 amino acids in length) which are then loaded on to MHC complexes and transported to the cell surface. The peptide fragments are bound within the major clefts of the MHC molecules and are recognized by the T-lymphocytes (CD8<sup>+</sup> T cell for MHC class I and CD4<sup>+</sup> T cell for MHC class II) via its clonotypically unique TCR<sup>[15]</sup>.

## Cross priming of T-lymphocytes

The mere encounter of a cytotoxic T-cell and a cognate foreign antigen is not enough for antigen recognition and expansion. Additional signals that arise out of receptor-ligand interaction between the T-lymphocytes and APCs are necessary for an active immune response to occur and is called cross priming of T-cells. DCs are called “immature” when they have not encountered antigen yet but have a higher capacity for phagocytosis<sup>[16]</sup>. Immature DCs are not capable of causing T-lymphocyte expansion but can produce immune tolerance when presenting self-antigens by causing T cell deletion or induction of regulatory T-lymphocytes<sup>[11,16,17]</sup>. Maturation of DCs occur in the lymph nodes and within areas of lymphocyte predominance thus increasing the chances of lymphocyte encounter and cross priming. Productive encounter of the TCR of CD8<sup>+</sup> or CD4<sup>+</sup> lymphocytes with p-MHC-I or p-MHC-II complexes respectively on the mature DCs (first signal) requires the ligation of lymphocyte receptor CD28 of the CD8<sup>+</sup> lymphocytes with its ligand (CD86) on DCs (the second signal or co-stimulation)<sup>[18-20]</sup>, as CD-28 deficient mice frequently have deficient T-cell responses<sup>[21]</sup>. The interaction of CD40 on CD4<sup>+</sup> lymphocytes with the CD40-L on the mature DCs is required for proper priming of the CTLs via CD28 and CD86<sup>[17]</sup>. Around the same time as the CD28-CD86 interaction, upregulation of the inhibitory receptor, cytotoxic T-lymphocyte antigen-4 (CTLA4), occurs and engages with CD80 (B7-2) (with a higher affinity compared to CD28) on DCs resulting in dampening of the T-cell response to prevent excessive immune reaction to antigen stimulus<sup>[22]</sup>.

## CD8<sup>+</sup> lymphocyte subsets and immunologic memory

The CD8<sup>+</sup> cytotoxic T-cells are essential for killing viral, protozoal, intracellular bacteria organisms and has a key role in preventing tumor growth and eradicating established tumors<sup>[23]</sup>. It also plays a significant part in mediating effectiveness of standard cytotoxic therapies for cancer<sup>[24,25]</sup>. In the context of eliminating microorganisms and tumor control, its cytotoxic effects are mediated via (1) perforin and granzymes through induction of caspases (identified by the expression of CD107a on degranulating cells); (2) the Fas/ Fas Ligand; (3) cytotoxicity aimed at tumor stromal cells including tumor vasculature; (4) secretion of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) that in turn induce tumor cell senescence; and (5) an anti-angiogenic effect by targeting tumor associated macrophages and secretion of IFN- $\gamma$  (a known inhibitor of tumor angiogenesis)<sup>[23]</sup>. Encounter of naïve CD8<sup>+</sup> T cells with p-MHC I complex on DCs results in a series of events grouped into three phases; (1) a multi-log clonal expansion of CTLs with capabilities of peripheral tissue homing, release of effector cytokine, and consequent cytotoxicity; (2) a contraction or “death” phase when there is rapid apoptosis of antigen-specific T-cells; and (3) development of long-lived antigen-specific cells that represent “memory” cells which reside in the peripheral lymph nodes (central memory cells) or can home in into the peripheral tissues where infection/tumor exist (effector memory cells)<sup>[26,27]</sup>. The attributes of these memory cells encapsulate the hallmarks of immunologic memory; increased precursor frequency compared to naïve lymphocytes, capacity for antigen-independent renewal in response to cytokines (IL-7, IL-15, and IL-21), procurement of effector functions and clonal expansion upon re-challenge providing long-term protection of the host from future pathogens or tumor recurrences<sup>[26]</sup>. Immunophenotypes of the different CD8<sup>+</sup> T cells are listed in Figure 1<sup>[27]</sup>. Naïve CD8<sup>+</sup> T cells express C-C chemokine receptor type 7 (CCR7) and CD62L to allow homing to lymphoid tissues<sup>[26,27]</sup>. Central memory CD8<sup>+</sup> lymphocytes are antigen-experienced cells that express CCR7 and CD62L allowing them to easily extravasate through



**Figure 1.** Phenotypic and function changes in CD8+ T cells following antigen (Ag) stimulation<sup>[27]</sup>

venules to populate T-cell zones within regional lymph nodes and provide protection against systemic antigen re-challenge<sup>[26,27]</sup>. These cells also have the ability to migrate to secondary lymph nodes by virtue of these two markers and secrete IL-2. Effector memory cells on the other hand, lose expression of these two molecules allowing them to migrate to peripheral tissues and protect against a peripheral challenge.

## TCRs

The TCR is a membrane bound receptor consisting of two polypeptide heterodimers ( $\alpha\beta$  or  $\gamma\delta$ ) connected by a disulfide bond and anchored to the membrane via a protein complex called CD3<sup>[28]</sup>. In the  $\alpha$  chain, the constant region is followed by the J region whereas in the  $\beta$  chain there is an intervening D region between the C and J regions. The complementarity determining region 3 (CDR3) (peptide region of 15 amino acids) consists of the VJ junction in the  $\alpha$  chain and the VDJ junctions in the  $\beta$  chain<sup>[28]</sup>. The CDR3 region confers the area of antigen contact and specificity for the TCR and is created by random joining of the V (54 regions), D (2 regions), and J (13 regions) genes. Further specificity is provided by random insertion and deletion of nucleotides in the V-D, V-J, and D-J junctions of the CDR3 domain during somatic recombination<sup>[28]</sup>. A similar process occurs with the  $\alpha$  chain without the D region. The TCR  $\alpha$  and  $\beta$  genes are located on chromosome 14 and 7 respectively.

## ADOPTIVE T CELL THERAPIES WITH TOTAL TUMOR RNA DC VACCINES IN CHILDREN WITH PRIMARY CNS TUMORS

Our lab has expertise in the development of *ex vivo* “educated” adoptive T-cell transfer along with total tumor RNA (ttRNA) DC vaccines for the treatment of brain tumors in the context of either MA or non-myeloablative (NMA) conditioning regimens.

### Neoantigen load and immune landscape of common pediatric brain tumors

In the last few years, advances in next generation sequencing in pediatric brain tumors has yielded a wealth of knowledge on the molecular landscape of various tumors including low grade gliomas, medulloblastomas, malignant gliomas (including diffuse pontine gliomas), ependymomas, and atypical teratoid rhabdoid tumors<sup>[29]</sup>. However, the overarching theme from these studies is that while actionable mutations do exist in all these tumors, the mutational load is significantly much lower than carcinogen-induced adult malignancies (non-small lung cancer or melanomas) except in the case of mismatch repair deficiency (MMR) induced malignant gliomas in children<sup>[30,31]</sup>. Unrepaired DNA damage in these tumors due to the somatic or germline mutations of *MSH1*, *MSH2*, *MSH6*, or *PMS-2*, *POLE*, or *POLD1* genes results in accumulation of an inordinate number of additional non-synonymous mutations resulting in a high neoantigen load and increased immunogenicity against these tumors due to lack of induction of central tolerance<sup>[30-32]</sup>.

Recent studies of the immune landscape of pediatric brain tumors demonstrates a tumor micro-environment that is variable across different tumor types. In an immune assay of 91 gliomas [glioblastoma multiforme (GBM) 68 and pilocytic astrocytoma 23] by immunohistochemistry (IHC), the CD8+ lymphocytes, CD56+ NK cells, and CD68+ macrophages were significantly higher in grade IV infiltrative glioma as compared to non-infiltrative grade I pilocytic astrocytoma<sup>[33]</sup>. Similarly, in a prospective randomized therapeutic trial in pediatric high grade gliomas using a backbone of bevacizumab (Avastin<sup>TM</sup>, Genentech corporation, San Francisco, CA) in patients receiving radiotherapy plus temozolomide (Temodar<sup>TM</sup>, Merck Co., Kenilworth, NJ), CD8+ infiltration (both perivascular and intratumoral) was highest in both MMR deficient malignant gliomas (four cases with somatic *POLE* or *POLD1* mutations; median mutation count of 4848, range 2197-5332) and anaplastic pleomorphic xanthoastrocytomas (with *BRAF* alterations)<sup>[32]</sup>. In this same study, RNA-sequencing data in a subset of samples revealed CD8 T cell effector/T cell signature that correlated with CD8+ T cell infiltration by IHC. This signature was particularly prominent in tumors with mitogen activated protein kinase pathway activation (including *BRAF v600e*, *NF-1*, and *FGFR1* mutations and *NTRK2* translocation). However, the histone mutant mid-line tumors (carry *H3F3A* mutations) had notable absence of immune infiltrates<sup>[32]</sup>. An IHC analysis of the antigen processing machinery (APM) in astrocytic tumors (4 each of grade I-IV gliomas) revealed down regulation of the APM proteins LMP-2, TAP1, and  $\beta$ 2 microglobulin without change in the surface human leukocyte antigen (HLA)-class I protein expression<sup>[34]</sup>. In 26 medulloblastoma samples, Vermeulen *et al.*<sup>[35]</sup> found CD3+ T cell intratumoral and/or perivascular in distribution. The number of CD3+ tumor infiltrating lymphocytes (TILs) was at a median of 23.5 per 2 mm<sup>3</sup> of tumor tissue. The phenotype was predominantly CD8+ T cells (52%) followed by CD4+ (35%) and CD4+CD25+ Fox P3 (2.5%) regulatory T cells. The number of TILs was not different between histologic or molecular subtypes of this tumor. There was also low CTL activation evidenced by only a small percentage of T-cells expressing granzyme B (3.9%; maximum 35%). In addition, decreased cell activation was attributed to a complete lack of expression of MHC class I on tumor cells (HLA-A and B) and CD1 d. Down regulation of MHC class I complex in medulloblastoma has similarly been reported in other studies<sup>[36,37]</sup>. One study of 10 primary medulloblastoma samples observed that while down regulation of class I molecules and associated proteins of APM machinery was found in these samples, HLA class I restricted tumor antigen specific CTLs that were generated by stimulation with DCs containing tumor mRNA, were able to effectively lyse medulloblastoma cell lines in a HLA-restricted manner, suggesting that down regulation or absence of MHC class I molecules or APM did not impact on tumor recognition by CTLs<sup>[37]</sup>. In addition, tumor cells expressed serpins (granzyme inhibitors) including serpin B1 and serpin B4 as additional means of immune evasion. The immune environment in ependymomas has been shown to determine patient prognosis between the two recently described molecular groups (Group A and B)<sup>[38,39]</sup>; in the unfavorable group A tumors, the gene expression pattern has more cell infiltration and immune gene signatures that indicate an immunosuppressive environment; in group B tumors there exists more of an immune stimulating response possibly predicting for a better prognosis in patients with these tumors both at initial diagnosis and following recurrence<sup>[40]</sup>. Furthermore, recurrent tumors from group A had higher expression of genes related to inflammation and immunoregulatory function and recurrent group B tumors had antiviral and adaptive



immune gene signatures<sup>[40]</sup>. In keeping with these gene signatures, group B recurrent tumors had higher infiltration of CD4+ and CD8+ T cells. When group A and group B tumors from diagnosis were evaluated for secretion of immune cytokines following stimulation, group B tumors secreted higher amounts of TNF- $\alpha$  (2.7 fold), IFN- $\gamma$  (5.3 fold), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (5.1 fold)<sup>[40]</sup>. In contrast to ependymomas, the tumor microenvironment of diffuse pontine glioma, a midline tumor with a dismal prognosis, is predominantly populated by CD11b+ macrophages which contain scant CD3+ T-lymphocytes; moreover, in contrast to malignant glioma, diffuse intrinsic pontine glioma *ex-vivo* cell cultures (patient-derived) release significantly less cytokines<sup>[41]</sup>.

### **TtRNA DC vaccines in pediatric brain tumors**

DC vaccines used in cancer are of four main categories and include peptide vaccines, cellular vaccines (tumor or immune cells), viral vector vaccines, and nucleic acid (DNA and RNA) vaccines<sup>[42,43]</sup>. Our lab has shown the use of RNA-pulsed DCs to be a versatile platform for activating tumor-specific T cells *in vitro* and *in vivo* in several murine and human systems. Several clinical trials have been conducted demonstrating the feasibility and safety of tumor lysate or RNA-pulsed DCs in human patients<sup>[44-46]</sup>. While specific tumor-associated antigens such as carcinoembryonic antigen, telomerase reverse transcriptase, melanoma antigens, epidermal growth factor receptor variant III (EGFRvIII), and human cytomegalovirus (CMV) phosphoprotein 65 (pp65) have all been successfully utilized in trials as either peptide vaccines or RNA-encoded antigens in DCs<sup>[46-48]</sup>, studies have demonstrated that the majority of endogenous anti-tumor immune responses in patients with malignancy are against unidentified, patient-specific antigens<sup>[49]</sup>. While use of ttRNA pulsed DCs to expand tumor-specific lymphocytes allows for these patient-specific antigens to be targeted, sufficient tumor tissue for clinical-scale vaccination is not always readily available. Our laboratory has utilized amplification of ttRNA with reverse-transcriptase primed polymerase chain reaction to generate cDNA library templates encoding for the antigenic content of tumor cells from as few as 500 starting tumor cells. Through inclusion of a T7 RNA polymerase binding site in the 5' primer used for amplification, ttRNA can be readily generated through *in vitro* transcription after cDNA amplification. Using such techniques, we have been able to generate enough RNA for clinical DC vaccine preparations from colorectal tumors, renal carcinoma, and pediatric and adult brain tumor specimens using excess tumor material harvested during surgical resection<sup>[50-52]</sup>.

While expansion of tumor cells using *in vitro* culture is feasible, primary brain tumor cells are often difficult to propagate and gene expression microarray analysis has demonstrated that most tumor specific genes expressed *in vivo* are not recapitulated within *in vitro* propagated tumor cells. Furthermore, we have demonstrated in murine intracranial glioma models that a significant shift in brain tumor gene expression is induced in response to host anti-tumor immunity<sup>[53]</sup>. This strongly suggests that the antigenic content of tumor cells propagated *in vitro* will be significantly different than *in vivo* propagated tumors, and thus the relevance of *in vitro* propagated tumors as an antigenic source for immunotherapy is questionable. This observation prompted us to investigate the capacity to amplify the RNA content of tumor cells isolated directly from surgically resected malignant glioma specimens to utilize an antigenic source more representative of the antigens expressed within patients' tumor cells *in vivo*. Based on current clinical protocols utilizing ttRNA pulsed DCs<sup>[54]</sup>, it is possible to produce up to 750  $\mu$ g of amplified tumor mRNA per patient. We have successfully amplified tumor mRNA to clinical scale (over 1mg) from as few as 500 astrocytoma cells from resected human glioma specimens from adult and pediatric brain tumors. Enrichment of tumor antigens can be done using subtractive hybridization of excess pooled normal brain RNA from tumor RNA prior to amplification and *in vitro* RNA synthesis and verification of enrichment of tumor-associated genes by comparative real-time PCR.

Once enough ttRNA is obtained, it can then be introduced via electroporation (300 V for 500  $\mu$ secs) into immature DCs derived from patient derived peripheral blood monocytes obtained via a peripheral blood mononuclear cell (PBMC) collection via apheresis. Differentiation of monocytes into DCs is achieved in *in vitro*



cultures under the influence of cytokines including GM-CSF and IL-4 for 7 days. The brief electrical current used during electroporation is enough to create a reversible breach in the cell membrane for the tRNA to rapidly enter the cytoplasm before degradation of the RNA. Electroporated tRNA results in better translation to tumor proteins in the cytoplasm of DCs and induction of tumor immunity in the host<sup>[42]</sup>. Although such immature tRNA containing DCs (tRNA DCs) can be administered as vaccine<sup>[55]</sup>, induction of immune responses is vastly inferior compared to mature tRNA DCs; maturity can be achieved by combination of cytokines including IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , and IL-6. Once matured, tRNA DCs can be frozen in aliquots of  $1 \times 10^7$  cells for clinical use.

### **Dose and route of administration of tRNA DC vaccine and immunoadjuvants**

While studies in adult cancers have shown that efficacy of monocyte derived tRNA DC vaccines might be dose dependent, larger dose vaccines do not necessarily produce proportional T cell responses but might rather dampen them by inducing immune tolerance<sup>[56]</sup>. In fact, pre-clinical studies have shown that as few as 85 mature DCs can produce adequate T-cell responses and ideally should be less than  $5 \times 10^6$  cells per dose<sup>[56,57]</sup>. Several routes of administration have been tried including intravenous, intradermal, and intranodal, injections<sup>[56]</sup>. Most vaccine cells end up in kidneys, lung, and spleen when given intravenously (as assessed by radiolabeling studies) and intranodal administration does not result in major distribution of DCs to other regional lymph nodes<sup>[56]</sup>. The skin is an ideal site for vaccination due to its rich plexus of blood vessels and lymphatics that coalesce and drain into the regional inguinal lymph nodes [also called vaccine site draining lymph nodes (VDLN)]. Although only 4% of an administered intradermal dose of vaccine reaches the VDLN (the rest either degrading locally or engulfed by macrophages)<sup>[57]</sup>, the number of mature DCs that migrate is still within the realm of less than  $5 \times 10^6$  cells when a total of a 10 million ( $1 \times 10^7$ ) cells are given with each vaccine dose. DC function and migration can be improved with immunoadjuvants<sup>[46,47]</sup>. The *ex vivo* maturation of immature DCs (which lack the capacity to migrate well) with a cytokine cocktail including IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , and IL-6, results in increased expression of the CCR7 and activation of the CCR7/C-C chemokine ligand type 21 (CCL21) mediated axis of migration of the DCs to the draining lymph nodes. In addition, co-stimulatory molecules expression is improved resulting in better cross-presentation and lymphocyte expansion in the regional nodes. Our laboratory has also shown that pre-conditioning of the one vaccine site with a recall antigen such as tetanus-diphtheria toxoid followed by bilateral administration of DCs pulsed with CMV pp65 RNA in patients with malignant glioma (which express pp65 in over 90% of tested samples) results in improved DC migration and improved survival as compared to control patients who receive only the pp65 DC vaccine<sup>[46]</sup>. A parallel experiment in mice confirmed these findings in a manner dependent on the increased systemic release of the chemokine CCL3<sup>[46]</sup>.

GM-CSF, a potent stimulant of the bone marrow granulocyte-monocyte progenitors, is secreted by endothelial cells, fibroblasts, and lymphocytes<sup>[58]</sup>. The cytokine can cause DC maturation on its own including expression of co-stimulatory molecules, induce CCR7 and migration towards CCL9, IL6 and TNF- $\alpha$  secretion and hence lymphocyte stimulation and expansion. In the context of immunotherapy, GM-CSF can be given either parenterally at a dose of 250  $\mu\text{g}/\text{m}^2/\text{day}$  for 5-7 days or pre-embedded in the vaccine using a significantly lower dose (75-150  $\mu\text{g}$  per vaccine) to minimize possible systemic adverse reactions with parenteral administration as well as minimize mobilization of immunosuppressive myeloid-derived suppressor cells (MDSCs) from the bone marrow with higher doses<sup>[58,59]</sup>. Cellular vaccines can also be genetically modified to secrete GM-CSF in the local milieu<sup>[60]</sup>. It should be noted that GM-CSF hypersensitivity due to autoantibody formation has been reported to occur when given with a vaccine<sup>[61]</sup>. At our institution tRNA-DC vials contain  $5.7 \times 10^6$  cells in 1mL. The final tRNA-DC product is a patient-specific single-dose syringe containing a total dose of  $1 \times 10^7$  cells formulated in 400  $\mu\text{L}$  of preservative free saline. The administered volume is 0.4 mL embedded with 150  $\mu\text{g}$  of GM-CSF. Half the dose is administered intradermally into each thigh about 5 cm below inguinal ligament. Patients in our clinical trials receive at least three biweekly vaccines with the first dose administered along with ALT infusion.

### Ex-vivo T cell expansion

The discovery in 1976 of IL-2, a T-cell growth factor, in the supernatant of phytohemagglutinin-activated human lymphocytes<sup>[62]</sup>, facilitated *ex-vivo* expansion of autologous T-cells without loss of effector function as well as the parenteral use of this cytokine to induce T-cell proliferation in the host<sup>[3]</sup>. Using this approach, impressive responses, albeit transient, were seen with bulky tumors in patients with lymphomas and metastatic melanomas. In a search of reactive T-cells and cognate tumor antigens, it was found that certain tumors, especially melanomas, harbored reactive lymphocytes in the tumor stroma that were of both CD4+ and CD8+<sup>[63]</sup>. These cells provided a rich source of possible TILs that if harvested, expanded *ex-vivo*, and reinfused could provide better therapeutic benefit<sup>[63]</sup>. Hence, TILs obtained from a resected melanoma and injected into the same host resulted in objective tumor regressions<sup>[63]</sup>. However, T-cells administered following such *ex-vivo* TILs expansion did not survive more than a few days due to immunosuppressive factors related to the tumor<sup>[63]</sup>. This obstacle was circumvented by using host NMA lymphodepletive conditioning using cyclophosphamide and fludarabine immediately prior to TIL transfer that led to increased longevity of the infused T cells and more oligoclonal expansion directed at specific antigenic epitopes (see later under “lymphodepletion with chemotherapy”)<sup>[64]</sup>. While TIL expansion and transfer can theoretically be possible in many human tumors with T-cell infiltration, it appears to be particularly suitable in melanomas due to high mutation burden and immunogenicity, characteristics of a carcinogen - induced tumor.

While it seems attractive to employ utilize this strategy in children with brain tumors, the lack of a prominent lymphocyte infiltrate in such tumors makes this an unsuitable approach in this patient population. However, the approach in our lab involves using ttRNA DCs to “educate” and expand naïve autologous lymphocytes *ex vivo* and reinfuse these lymphocytes (ttRNA-xALT) into the host after lymphodepletive conditioning using either MA or NMA chemotherapy followed by hematopoietic stem cell (HSC) rescue and sustained the expansion of these “educated” lymphocytes by regular administration of ttRNA DCs. We have demonstrated the capacity to induce anti-tumor lymphocytes *in vitro* and *in vivo* using tumor RNA-pulsed DCs from a variety of human and murine tumors, including malignant brain tumors<sup>[50,52,55,65-73]</sup>. We have explored the capacity to enhance the yield of antigen-specific T cells *ex-vivo* through a rapid expansion protocol (REP) developed at the National Cancer Institute for expansion of melanoma-reactive tumor-infiltrating lymphocytes<sup>[74]</sup>. The REP employs irradiated allogeneic PBMCs as feeder cells, low-dose anti-CD3 monoclonal antibodies (OKT3), and IL-2 for the rapid expansion of activated lymphocytes in culture<sup>[74]</sup>. This process has the capacity to expand lymphocytes stimulated by RNA-pulsed DCs 200-500 fold and is possible to achieve greater than  $1 \times 10^{10}$  T-cells with an input of  $1 \times 10^8$  activated lymphocytes. The addition of IL-21 and IL-7 during co-culture of T cells with RNA-pulsed DCs prior to REP leads to a greater generation of central memory antigen-reactive T cells which have been shown to be superior in anti-tumor efficacy.

### Lymphodepletion with chemotherapy augments immune responses

After episodes of lymphopenia, host recovery ensues before regaining normal lymphocyte counts<sup>[75]</sup>. After profound lymphopenia<sup>[75,76]</sup>, there may be an increase in cytokines (i.e., IL-7, IL-15) which can induce lymphocyte differentiation into effector memory T cells imbued with memory recall against target antigens<sup>[77]</sup>. However, under these conditions, competition for homeostatic cytokines remains a potent barrier for lymphocyte proliferation<sup>[75]</sup>, which may allow antigen-specific B- or T-cells (generated through vaccination) to predominate during recovery from lymphodepletive therapy; data to support this has been shown in murine model<sup>[78,79]</sup> and in humans<sup>[80]</sup>. The predominance of these cell types may augment anti-tumor response<sup>[78,79,81]</sup>, but may theoretically also precipitate autoimmunity<sup>[82,83]</sup>.

Rosenberg *et al.*<sup>[84]</sup> used NMA lymphodepletion to sustain and augment these antigen specific memory T cell subsets against refractory malignancies, and achieved impressive responses in patients<sup>[84-88]</sup> despite autoimmunity<sup>[64,86]</sup>. Under these NMA contexts, adoptively transferred T cells proliferate intensely following lymphopenia and comprise the bulk of a T cell repertoire in a treated host, which persists for months after cell transfer<sup>[64,89]</sup>. Moreover, disease regression strongly associates with the amount and persistence of these

antigen-specific T cells<sup>[84-88,90]</sup>. This anti-tumor effect can also be significantly enhanced by MA conditioning regimens coupled with autologous HSC support. Interestingly, HSCs were found to confer an enhancing effect on the *in vivo* expansion and persistence of tumor-specific lymphocytes transferred simultaneously with HSCs independent of the effects of lymphopenia<sup>[91]</sup>. Pursuant to these findings, Rosenberg and colleagues proceeded to evaluate MA conditioning regimens coupled with peripheral blood stem cell (PBSC) rescue in patients with malignant melanoma receiving ACT. ALT was administered in the peri-transplant period within 24 h of PBSC infusion and was feasible and safe in humans<sup>[92]</sup>. As demonstrated in murine studies, this conditioning regimen enhanced anti-tumor responses in patients with refractory metastatic disease, resulting in increased objective clinical responses from 30%-50% of patients with NMA regimens to over 70% in patients receiving MA conditioning coupled with PBSC infusion.

The mechanisms by which lymphodepletion leads to an enhancement of immune responses in humans are not well elucidated but elegant murine studies have implicated the following important processes: (1) increased in production of homeostatic cytokines such as IL-7 and IL-15 that drive lymphocyte proliferation<sup>[93]</sup>; (2) decreased competition with adoptively transferred tumor-specific lymphocytes through removal of “cytokine sinks” consisting of host lymphocytes and NK cells that decrease the bioavailability of growth factors<sup>[94]</sup>; (3) removal of CD4+CD25+Fox P3+ tregs that attenuate anti-tumor immunity<sup>[95]</sup>; (4) increased toll-like receptor agonistic signals and inflammatory cytokines through release of gut microbial antigens such as endotoxin during damage to gut endothelium by MA therapy<sup>[96]</sup>; and (5) direct enhancing effects of HSC transplant on the *in vivo* expansion and function of adoptively transferred lymphocytes<sup>[97]</sup>.

### **HSCs augment immune responses during ttRNA-DC + ttRNA-xALT therapy**

MA chemotherapy with HSC rescue is frequently used in children with primary brain tumors including medulloblastoma, other central primitive neuro-ectodermal tumors (PNETs), and in malignant glioma both at diagnosis as well as at recurrence<sup>[98]</sup>. The HSC rescue serves to repopulate the bone marrow and recovery following myeloablation and additionally helps in the reconstitution of the host immune system. Since MA conditioning with HSC rescue is used in our adoptive T-cell therapy protocols, we wanted to explore potential immune-modulatory effects of HSC in addition to its role in recovery from lethal bone marrow damage. It has been previously shown that HSCs can promote the expansion and function of CD8+ T-cells by secreting homeostatic cytokines IL-7 and IL-15<sup>[91]</sup>. In a pre-clinical highly invasive chemotherapy and radiotherapy resistant orthotopic glioma mouse model (KR158B glioma), administration of MA conditioning + HSC + x-ALT + ttRNA DC (× 3 vaccines) produced significantly improved survival and cures in 30% of animals as compared to tumor bearing controls (no treatment, MA conditioning alone, MA + HSC + x-ALT only, or ttRNA DC only)<sup>[99]</sup>. The x-ALT cells were syngeneic splenocytes harvested from tumor antigen-primed mice and expanded *ex-vivo* with ttRNA DCs in the presence of IL-2. The HSCs were found to migrate into the tumor and attract activated T-cells into the tumor. Correlative studies found that tumor elaborating C-X-C motif chemokine 12 (CXCL12) attracted the HSCs into the tumor by expressing the cognate receptor C-X-C motif chemokine receptor 4 (CXCR4). T-cells (both CD4+ and CD8+) were attracted into the tumor milieu following secretion of CCL3 by the HSCs<sup>[99]</sup>. In addition, maintenance DC vaccines were crucial in maintaining this immune response<sup>[99]</sup>. In probing the role of HSCs further in inducing immune responses, it was found that in addition to attracting effector T-cells into the tumor microenvironment, HSC infusion precipitated production of activated CD86+CD11c+MHC class II+ cells consistent with a DC phenotype in this tumor milieu and replacement of host MDSCs<sup>[100]</sup>. This was attributed to the differentiation of the HSCs into DC under the influence of T-cell secreted IFN-γ<sup>[100]</sup>.

### **Pre-clinical and clinical studies of ttRNA DC vaccine +/- x-ALT in primary CNS tumors**

In our laboratories and those of others, systemic immunization using DCs co-cultured with uncharacterized tumor homogenate<sup>[44,101]</sup>, whole tumor RNA<sup>[55]</sup>, unidentified peptides eluted from tumor cells by gentle acid washing<sup>[102]</sup>, or a distinct peptide encompassing the tumor-specific *EGFRvIII* mutation<sup>[103]</sup> have been shown to induce humoral and cell-mediated systemic immune responses and to prolong the survival of mice with intracranial brain tumors. We have used a strain of mice (VMDk) that is susceptible to experimental

autoimmune encephalitis to demonstrate the safety and efficacy of ttRNA pulsed DCs in mediating potent antitumor immune responses and regression of established tumor that has prolonged survival in treated animals without causing inflammatory reactions in the CNS<sup>[65,66]</sup>. Our group has previously demonstrated the efficacy of adoptive cell therapy employing tumor-specific T cells generated from bone marrow-derived DCs pulsed with ttRNA against intracranial glioma<sup>[99]</sup>.

### **Phase 1 study in pediatric patients with recurrent medulloblastoma and primitive neuroectodermal tumors (reMATCH trial, NCT01326104)**

Using a strategy similar to adoptive T-cell therapy pioneered by Dr. Rosenberg at the National Cancer Institute in patients with metastatic melanoma, we recently completed a phase I study of ttRNA - pulsed DC vaccine + ttRNA-xALT following MA or NMA conditioning in 10 patients with recurrent PNET and GBM (medulloblastoma 8, pineoblastoma 1, and GBM 1)<sup>[104]</sup>. All patients had tumor resection/biopsy to confirm recurrence and obtain tissue for vaccine preparation. PBMCs were collected following surgical recovery for DC preparation and T-cell expansion. Patients then received either NMA conditioning with cyclophosphamide + fludarabine ( $n = 9$ ) or MA conditioning with carboplatin + thiotepa + etoposide with PBSC support ( $n = 1$ ) followed by xALT in one of two dose levels;  $3 \times 10^6$  cells/kg ( $n = 3$ ) and  $3 \times 10^7$  cells/kg ( $n = 7$ ). All patients received at least 3 doses of ttRNA DCs once every 2 weeks at a  $1 \times 10^7$ /kg per dose. The median number of vaccines given was 3 (range, 3-9). Of 8 evaluable patients for dose-limiting toxicity by receiving ttRNA x-ALT and at least one dose of ttRNA DC, there were no dose limiting toxicities associated with ttRNA DCs + ALT. Toxicities that were possibly attributable to immunotherapy included grade I rash ( $n = 1$ ) and a transient grade III elevation of serum alkaline phosphatase ( $n = 1$ ) 3 months after the 3rd dose of ttRNA DC. Median time to progression in 9 patients from 1st ttRNA DC + x-ALT administration was 5 months (range, 2-24) and median survival 13 months (range, 2-46+ months). In a recurrent tumor with a dismal prognosis, 5 patients survived for > 20 months following first dose of ttRNA DC + x-ALT. Three of 10 patients are currently alive; 2 patients who relapsed 12 and 24 months respectively following immunotherapy but currently alive following additional salvage therapies at 45+ and 46+ months. One additional patient with Gorlin's syndrome and recurrent medulloblastoma is currently alive 42+ months following immunotherapy (received a total of 9 doses of ttRNA DCs) and never received radiotherapy either at diagnosis or at relapse. Measurement of inflammatory cytokines (IFN- $\gamma$ , TNF, IL-6, IL-8, and IL-17A) was elevated in general following MA or NMA chemotherapy. Lymphocyte recovery occurred in all patients at variable intervals. Using next-generation sequencing (NGS) we evaluated TCR-V $\beta$  clones in the PBMC samples from all patients at baseline and at regular intervals during and post immunotherapy. Clonal diversity during recovery was higher in patients with prolonged survival (> 20 months;  $n = 5$ ). Clonal hyper-expansion and persistence appeared to be higher in patients with prolonged survival and reached significance at day 7 following x-ALT administration. One patient who received MA conditioning prior to x-ALT and 3 ttRNA DCs and is a long-term survivor at 46+ months, experienced massive and selective expansion of 4 tumor-reactive TCR V $\beta$  clones in the peripheral blood up to four months (16 weeks) post-treatment (Flores *et al.*<sup>[99]</sup>, 2018, submitted for publication). T-cells from one of these clones was tested for anti-tumor function against patient's ttRNA DCs. IFN- $\gamma$  secretion was measured to indicate recognition of cognate tumor antigen and found to be elevated compared to control ovalbumin-RNA containing DCs. This data suggests that expansion of productive frequency of TCR V $\beta$  family is potentially predictive of T cell clonal expansion within the larger family. Analysis of TCR V $\beta$  family expansion in peripheral blood of treated patients could be predictive of response to adoptive immunotherapy. We have subsequently enrolled 23 subjects (screened 34) in an ongoing multi-institutional phase II study in which our institution serves as the central GMP manufacturing facility for autologous cellular products.

### **Ongoing phase I studies in newly diagnosed malignant glioma and diffuse pontine glioma**

We have also initiated two additional upfront phase I clinical trials in children with newly diagnosed malignant glioma (ACTION trial, NCT03334305) and diffuse brain stem glioma (BRAVO trial,



NCT03396575) using the ttRNA DC vaccine + x-ALT platform with some modifications from the reMATCH trials. While the general strategy in both clinical trials is similar to reMATCH, dose-intensive temozolomide (temodar<sup>TM</sup>, Merck, Kenilworth, NJ) (TMZ) is used in these two studies as both adjuvant chemotherapy post standard chemo-radiotherapy (concurrent TMZ) and as a lymphodepletive agent prior to HSC and ttRNA DC + -x-ALT and during maintenance monthly ttRNA DCs. We have introduced a few changes in these two trials including (1) obtain autologous lymphocytes after 3 bi-weekly ttRNA DCs following chemo-radiotherapy; (2) *ex-vivo* expansion using REP; and (3) administer up to a total of 10 ttRNA DCs.

## CONCLUSION

The field of adoptive T-cell therapy using x-ALT + tt-RNA DCs in children with brain tumors is evolving and appears, in our preliminary experience, to have provided sustained benefit in a handful of patients with recurrent medulloblastoma without undue toxicity. It is obvious that success in a larger proportion of treated children is unlikely to equal what has been observed in adults with metastatic melanomas. This might be related to the differences in the degree of immunogenicity and mutational load seen in tumors like melanomas that is hard to match in pediatric brain tumors that appear to have an immunosuppressive microenvironment. It is intriguing to speculate whether the rare population of children with germline *p53* mutations with medulloblastoma<sup>[105]</sup> or MMR deficiency malignant gliomas<sup>[30]</sup> might be a more suitable population to evaluate this therapeutic approach given the exceptionally high mutational load in these tumors compared to wild type counterparts. For most pediatric CNS tumors with an immunosuppressed landscape as previously discussed due to decreased MHC class I expression, decreased or absent TILs, high programmed cell death-1 (PD-1) or programmed cell death ligand-1 (PD-L1) expression, or increased MDSC infiltration, significant refinements need to be made to improve immune responses and outcome. It is also entirely possible that utilizing this strategy in the upfront setting in treatment-naïve patients might provide better outcomes due to lack of prior treatment related immune suppression and minimal tumor burden. Radiotherapy, typically given at diagnosis, will further reduce tumor bulk and augment immune responses through multiple mechanisms<sup>[106]</sup>. The role of the microbiome in affecting outcome following immune checkpoint inhibitor therapy has been confirmed recently in pre-clinical studies of mice bearing melanoma and non-small lung cancer tumors<sup>[107,108]</sup>. Whether such optimization of fecal microbiome in patients receiving ALT will prove beneficial remains to be evaluated. Using NGS methodology and HLA-typing to improve prediction of MHC-I class and MHC-II class binding epitopes to create a robust neoantigen predominant transcriptome for electroporation into DCs<sup>[47]</sup>, nanoparticle vaccines<sup>[109]</sup>, and/or the use of immune checkpoint inhibitors<sup>[30,110]</sup> are other potential strategies to enhance efficacy in an adjuvant setting or relapse following x-ALT + ttRNA DCs. With these refinements and more this form of ACT promises to be an important therapeutic approach in the management of pediatric brain tumors.

## DECLARATIONS

### Authors' contributions

Conceived and wrote the manuscript: Gururangan S

Helped with critical review and revisions: Sayour E, Mitchell DA

### Availability of data and materials

Not applicable.

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None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.



## Ethical approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

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Review

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# Histone deacetylase enzymes and selective histone deacetylase inhibitors for antitumor effects and enhancement of antitumor immunity in glioblastoma

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## Abstract

Glioblastoma multiforme (GBM), which is the most common primary central nervous system malignancy in adults, has long presented a formidable challenge to researchers and clinicians alike. Dismal 5-year survival rates of the patients with these tumors and the ability of the recurrent tumors to evade primary treatment strategies have prompted a need for alternative therapies in the treatment of GBM. Histone deacetylase (HDAC) inhibitors are currently a potential epigenetic therapy modality under investigation for use in GBM with mixed results. While these agents show promise through a variety of proposed mechanisms in the pre-clinical realm, only several of these agents have shown this same promise when translated into the clinical arena, either as monotherapy or for use in combination regimens. This review will examine the current state of use of HDAC inhibitors in GBM, the mechanistic rationale for use of HDAC inhibitors in GBM, and then examine an exciting new mechanistic revelation of certain HDAC inhibitors that promote antitumor immunity in GBM. The details of this antitumor immunity will be discussed with an emphasis on application of this antitumor immunity towards developing alternative therapies for treatment of GBM. The final section of this article will provide an overview of the current state of immunotherapy targeted specifically to GBM.

**Keywords:** Glioblastoma, histone deacetylase inhibitors, antitumor effects, antitumor immunity

## INTRODUCTION

Glioblastoma multiforme (GBM) is the most prevalent primary malignancy in the central nervous system (CNS) in adults. GBM still remains incurable and thus continues to present a formidable challenge to both



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clinicians and researchers alike. Classified as a grade IV glioma by the World Health Organization (WHO)<sup>[1]</sup> this tumor's dismal survival rates are owed to its ability to recur following first-line treatment strategies such as surgical resection, radiation therapy, and chemotherapeutic agents - the current standard of care. This tumor also possesses the ability to evade current first-line treatment strategies through the development of multiple resistance mechanisms, which it employs to recur despite initial response to these strategies<sup>[2]</sup>. GBM is also simply called glioblastoma. The most resistant glioblastoma cells, also known as glioblastoma stem cells (GSCs), which remain alive following first-line therapy have employed resistance mechanisms and will go on to form recurrent glioblastomas. These tumors are more difficult to treat as they confer resistance to first-line treatment strategies, requiring alternative therapies. The agents currently needed to combat these recurrent glioblastomas are lacking. As such, developing novel therapeutic agents based on inquiries into the biochemical specificities and pathogenesis of these tumors has been a hotbed of research in recent years. Novel therapeutic agents have shown considerable promise in their developmental phases but have yet to replace the current standard of care. The current standard of care includes surgical resection, radiotherapy, and the chemotherapy using temozolomide (TMZ)<sup>[3]</sup>. Multiple avenues have been explored for potential therapeutic strategies. One particularly exciting avenue of research is immunotherapy, which harnesses the immune system to aid in abolishing the growth of glioblastoma<sup>[4,5]</sup>.

Immunotherapy has seen success in the clinical realm in recent years, a success that can be attributed to a more robust understanding of basic tumor immunology in order to aid the immune system in fighting a neoplastic process<sup>[6]</sup>. Previously, the lack of clinical efficacy in immunotherapy was due to the ability of many tumors to avoid recognition and therefore elimination by the immune system<sup>[7]</sup>. However, active research in this area into how the tumor evades the immune system has led to novel therapies in the fight against these pathologies, with cancer immunotherapy even being heralded as the "breakthrough of the year" roughly five years ago<sup>[8]</sup>. Most recently, immunotherapy specific to malignancies has been such an exciting breakthrough that Drs. James P. Allison and Tasuku Honjo have received the 2018 Nobel Prize in Physiology or Medicine for their contributions to the field of cancer immunotherapy and identification of the immune checkpoint proteins [e.g., programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1), cytotoxic T lymphocyte associated antigen-4 (CTLA-4)] that usually act as a brake on the immune system. For modulating the immune system, these therapies have employed multiple strategies including inhibiting immune checkpoints, expanding an existing immune system response, enhancing the immunological profile of solid tumors, natural killer (NK) cell/chimeric antigen receptor (CAR)-T cell modulation, and T regulatory (Treg)/myeloid suppressor cell modulation<sup>[9]</sup>. Immunotherapy, like any other new treatment modality heralded as panacea, ultimately has its limitations and downsides when being used to treat cancer. These limitations are especially evident with glioblastoma, as certain modalities of cancer immunotherapy (immune checkpoint inhibitors, CAR-T cell therapy, *etc.*) require continued research and further clinical trials if they are to be considered in the next step in the targeted glioblastoma therapy<sup>[10,11]</sup>.

A challenge specific to glioblastoma and a potential barrier to the application of immunotherapy to these tumors is the presence of the blood-brain barrier (BBB), which forms a protective coating around the brain made up of tight junctions between astrocytes. Traditional dogma had considered the brain to be an inaccessible site, due to rudimentary studies in the late 19th century, and early 20th century with dyes injected into the blood not showing up in the brain upon autopsy<sup>[12]</sup>. Years later, an extension of this experimentally derived dogma also assumed that the CNS was among many tissues to be an "immunoprivileged" site<sup>[13]</sup> largely derived from studies of grafts transplanted in the CNS that failed to be rejected when similarly grafted into other sites that were more immunologically accessible within the body. Additionally, the brain's lack of draining lymphatics, the apparent immunoincompetence of microglia (the brain's resident macrophages), and the assumption of CNS autoimmunity being a direct consequence from CNS antigen encounter by an immune cell cemented the idea of the brain being an inaccessible sanctuary away from the body's immune system<sup>[14]</sup>. However, today this is not believed to be the case. A physiologically functioning BBB is now believed to act as a communication center of sorts, passing (and responding to)

signals from the blood, regulating entry and exit of molecules from the blood and the CNS, and even changing as the somatic demands of the barrier changes<sup>[15]</sup>. This physiological barrier is often deregulated due to development of a brain malignancy such as glioblastoma, which again endorses the notion that tumors within the brain are inaccessible to the therapeutic agents as they cannot cross the BBB<sup>[16]</sup>. This fundamental change in understanding of how the BBB functions along with the rise of immunotherapy as a promising cancer treatment modality has opened wide the application of this therapy as a potential treatment for GBM<sup>[17]</sup>, which in the past has been extremely difficult to treat.

One specific modality of immunotherapy that has shown some promise in the treatment of GBM is the use of epigenetic modulators. Histone deacetylases (HDACs) play important roles in epigenetic changes and HDAC inhibitors as immunomodulatory agents have been useful in the preclinical arena to promote immune-mediated destruction of neoplastic cells in the CNS. These epigenetic modulators work to alter gene expression without alteration of the DNA sequences, through modulation of specific signaling cascades within the tumor<sup>[18]</sup>. In fact, one specific class of compounds that have currently shown promise in epigenetic modulation of GBM cells are the HDAC inhibitors<sup>[19]</sup>. This epigenetic approach towards cancer therapy involves tipping the balance between the activity of two different enzyme families, histone acetyltransferases (HATs) and HDACs. HATs have classically been involved in increasing gene expression, while HDACs have been associated with gene silencing. Mutations in HDAC enzymes have been linked to tumor development, due to the lack of inactivation of aberrant genes involved in the regulation of important cellular functions including cell proliferation, cell cycle regulation, and apoptosis<sup>[20]</sup>. Following the discovery of these dysregulated pathways in tumor cells, investigation into HDAC inhibitors has become an active area of research. Some of these agents had questionable efficacy when used as monotherapy against many human tumors, but when utilized in combination therapies with standard-of-care treatment regimens, they showed synergistic or additive effects<sup>[21]</sup>. In glioblastoma specifically, this treatment modality has demonstrated both induction of apoptosis and promotion of antitumor immunity<sup>[22]</sup> providing a potential method of immunotherapy directed against glioblastoma.

In this review article, we seek to examine the current understanding of HDAC enzymes, describe progress in the development of HDAC inhibitors being used to treat glioblastoma, and report other potential immunomodulatory agents and immunotherapy modalities with a potential to be directed to glioblastoma. As unvaryingly lethal as this tumor is, the potential of novel therapeutic agents must not be overlooked in HDAC inhibitors because any new therapy may provide a new chance at remission for glioblastoma patients who are in desperate need of novel approaches towards fighting their malignant condition.

## HDAC ENZYMES

HDAC enzymes serve as some of the most important effectors of epigenetic changes in the human body. First isolated from a calf thymus extract<sup>[23]</sup>, HDACs were found to catalyze the removal of acetyl groups from lysine residues of both histone and non-histone proteins, thereby effecting transcriptional changes within the cells<sup>[24]</sup>. This function of histone deacetylation was suspected to be caused by a complex of multiple enzymes, but early chromatography studies were unable to differentiate the function of individual enzymes that made up this complex. However, this state of understanding changed significantly following the cloning of the first HDAC enzyme in 1996 (aptly described as HDAC1 in the literature)<sup>[25]</sup>. This began a wave of research publications fully describing these enzymes and their functions. Today, there are 18 different human HDAC enzymes divided into two separate families and four classes based on their similarities to their yeast enzyme counterparts [Table 1].

All 18 HDAC enzymes belong to either the HDAC family or the silent information regulator 2 (Sir2) family, with the human versions of these enzymes being further subcategorized into the classes based on their similarities in amino acid sequence. HDAC1, HDAC2, HDAC3, and HDAC8 are all class I proteins with

**Table 1. Characteristics of the human histone deacetylase enzymes and their similarity to yeast proteins**

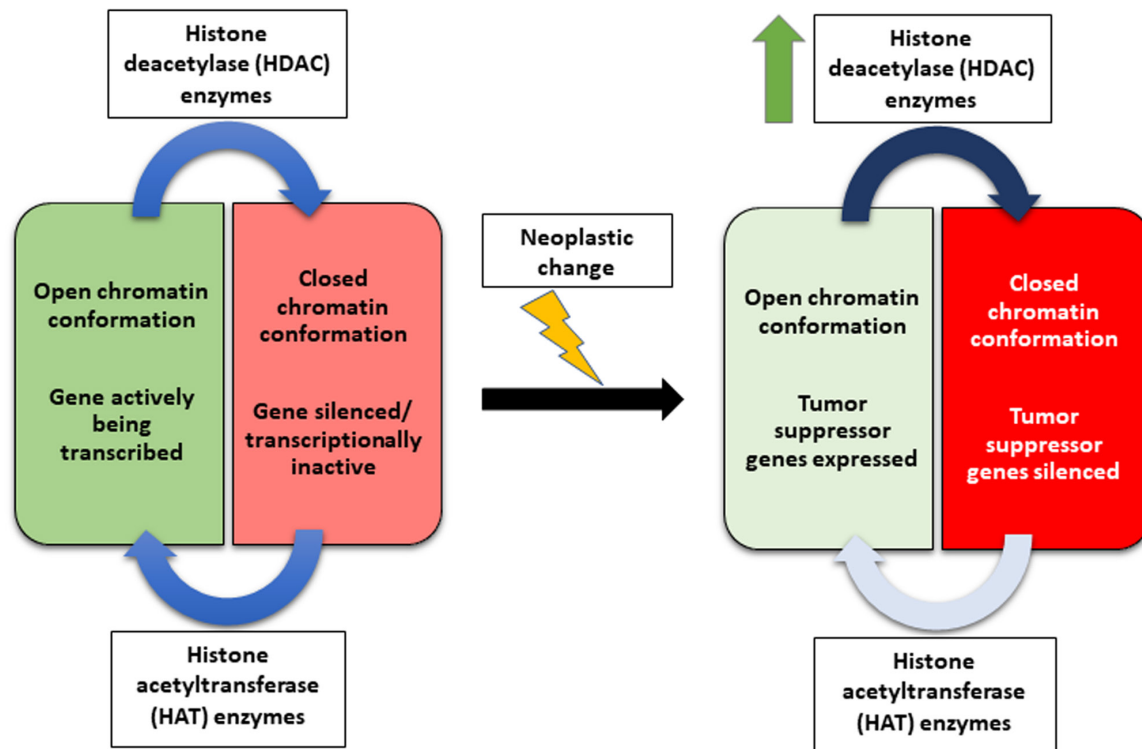
HDAC enzyme class	HDAC enzymes*	Protein family	Required catalytic cofactor	Resembled yeast protein sequence
I	HDAC1, HDAC2, HDAC3, and HDAC8	Histone deacetylase	Zn <sup>2+</sup>	Rpd3
II	HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10	Histone deacetylase	Zn <sup>2+</sup>	Hda1
III	SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7	Sir2 regulator	NAD <sup>+</sup>	Sir2
IV	HDAC11	Histone deacetylase	Zn <sup>2+</sup>	Class I and II HDACs

\*HDAC enzymes have been divided into four classes based on their similarity in sequence and function to well-described yeast proteins. Class I enzymes include HDAC 1, 2, 3, and 8 that belong to the classical HDAC family, require a Zn<sup>2+</sup> for their catalytic action, and are similar to the yeast protein Rpd3. Class II enzymes contain HDAC 4, 5, 6, 7, 9, and 10 that also belong to the classical HDAC family, also require a Zn<sup>2+</sup> for their catalytic action, and are similar to the yeast protein Hda1. Class III enzymes differ most significantly from their HDAC counterparts, containing SIRT 1, 2, 3, 4, 5, 6, and 7 that belong to the distinct Sir2 regulator family, require NAD<sup>+</sup> as an essential catalytic cofactor, and are similar to the yeast protein Sir2. Finally, class IV contains only HDAC11 that is also part of the classical HDAC family, requires a Zn<sup>2+</sup> for its catalytic action as well, and most resembles the class I and II HDAC enzymes. These enzymes are numbered in the order in which they were discovered. HDAC: histone deacetylase; SIRT: sirtuin

sequence similarity to a yeast protein, which is called the reduced potassium dependency 3 (Rpd3). HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 are all class II proteins with sequence similarity to the yeast protein histone deacetylase-A 1 (Hda1). Class I HDACs are ubiquitously expressed in all tissues while class II HDACs are tissue-specifically expressed<sup>[26]</sup>. Sirtuin is a word coined from its founding member Sir2 in the yeast *Saccharomyces cerevisiae*. Sirtuin 1 (SIRT1), SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7 in humans are all class III proteins with sequence similarity to the *S. cerevisiae* protein known as the Sir2. Finally, HDAC11 is the lone member of class IV and shares sequence similarity to both the class I and class II HDACs. These enzymes, for the most part, were numbered according to the order in which they were discovered.

HDACs in classes I, II, and IV are in the superfamily of proteins known as the arginase/deacetylase superfamily, which contains the arginase-like amidino hydrolases and the HDACs. The HDAC enzymes in classes I, II, and IV belong to the classical HDAC family and require a zinc ion (Zn<sup>2+</sup>) for their catalytic action to take place. HDACs in class III, however, belong to the deoxyhypusine synthase-like nicotinamide adenine dinucleotide (NAD)/flavin adenine dinucleotide-binding-domain superfamily of proteins, which contain the Sir2 proteins as well as many other sequence-similar enzyme families. In contrast to the classical HDAC family of enzymes, class III enzymes require NAD<sup>+</sup> as a cofactor for enzyme activity instead of a Zn<sup>2+</sup><sup>[27]</sup>. While there are subtle differences in the classification scheme of these enzymes, they play an essential functional role in maintaining the balance between histone acetylation and deacetylation. This balance ultimately mediates access of transcriptional machinery to the chromatin of the cell, with downstream consequences such as alteration in gene transcription. However, functionality of these enzymes is much more complicated than one HDAC per one histone (or non-histone) protein. These enzymes as a superfamily are biologically essential due to their opposition of the effects of HAT enzymes, where a defect in this balance leads to epigenetic changes in the aberrant tissue [Figure 1].

A change in the cellular balance between HAT and HDAC enzyme activity modifies gene expression and translation of mRNA transcripts into protein products. However, this cellular balance is very delicate and has been classically shown that “minor” histone modifications can greatly influence gene transcription<sup>[28]</sup>. In fact, in one genome-wide mapping study, HDACs were observed to be bound to chromatin at actively transcribed genes, but not silent genes<sup>[29]</sup>. These HDACs are believed to be able to reset active chromatin, silencing the gene after making the desired protein product by the cell. Additionally, non-histone proteins are also subject to cellular changes through acetylation. Noteworthy non-histone proteins that can cause great cellular change include transcription factors, chaperone proteins, viral proteins, and proteins involved in DNA repair, recombination, and replication<sup>[30]</sup>. These non-histone proteins have been implicated in essential cellular processes such as chromatin remodeling, cell cycle regulation, apoptosis, autophagy, and actin nucleation<sup>[31]</sup>. The HDACs have been implicated in pathology as well, where their dysregulation halts the repression of active genes in the cell, leading to an abnormal expression of certain protein products.



**Figure 1.** Histone acetyltransferase (HAT) and histone deacetylase (HDAC) in balance - physiologic vs. pathologic. In physiologic state, HAT enzymes and HDAC enzymes work in tandem to regulate gene transcription. HATs induce an open chromatin conformation (favoring gene transcription), which is counterbalanced by the action of HDACs that induce a closed chromatin conformation (favoring gene silencing). In pathologic state (e.g., neoplastic change) this balanced is tipped, favoring either an unregulated open chromatin conformation or an unregulated closed chromatin conformation. Schematically shown is an instance of an unregulated closed chromatin conformation due to a pathologic increase in HDAC enzymes. This unregulated, pathologic state may silence physiologic regulatory pathways in the cell, such as those protein products that regulate the cell cycle genes (e.g., tumor suppressor genes)

Alternatively, HDACs can also be overexpressed in abnormal tissue, leading to the silencing of regulatory genes [Figure 1]. Over the years, abnormal HDAC transcripts have been linked to multiple pathologies including neurological diseases, immune disorders, and a multitude of cancers<sup>[32,33]</sup>.

Cancer is a particular field where HDAC enzymes are heavily implicated, as there are correlations between somatic DNA mutations in histone-modifying enzymes and human malignancy<sup>[34]</sup>. One of the first examples of note was the discovery of a mutation in HDAC2, leading to microsatellite instability in those individuals with hereditary non-polyposis colorectal carcinomas<sup>[35]</sup>. The expression of HDAC transcripts has also been found to be variable in tumors when compared to normal somatic tissue, such that newer studies can link abnormal HDAC activity in 21 liquid and solid human tumors<sup>[36]</sup>. These changes in HDAC activity may lead to changes in histone acetylation status, thereby leading to increase in transcription of human oncogenes or suppression of tumor suppressor genes. Aberrant expression of HDACs has been shown to be correlated with a poor clinical prognosis<sup>[37]</sup>. These enzymes ultimately play an essential role in the body, providing a stabilizing force to the action of HATs and effecting epigenetic change. When researchers knew that these enzymes were often aberrantly expressed in tumors, they began setting their sights on understanding their roles in the pathogenesis behind one of the deadliest human cancers, glioblastoma.

## GLIOBLASTOMA AND DEREGLATION OF HDAC ENZYMES

HDAC enzymes may play a role in the tumorigenesis of glioblastoma through a yet-undetermined mechanism. HDACs are believed to be effectors of epigenetic changes observed in neoplastic tissue,

particularly glioblastoma, when compared to non-neoplastic tissue. Among the many epigenetic alterations observed in glioblastomas, changes in HDACs specifically present an opportunity to monitor the transcriptional status of the genome in these tumors. Preliminary evidence showed that class II and class IV HDACs display decreases in mRNA expression in glioblastoma when compared to other, more low-grade gliomas and normal brain tissues, and an increased amount of acetylation in histone protein H3<sup>[38]</sup>. Histone modifications are frequent epigenetic changes observed in tumor analysis<sup>[39]</sup>. Additionally, one large-scale sequencing study of the protein-coding genes in glioblastomas revealed mutations in two genes *HDAC2* and *HDAC9*<sup>[40]</sup>. While this handful of preliminary studies have shown that HDAC enzymes see a decrease in expression, other more recent studies have shown HDAC enzymes seeing an increase in their expression, further complicating the picture of expression of HDAC enzymes in glioblastomas.

In recent years, further cytologic examinations of tumor samples have revealed an ambiguous picture as to the expression of HDAC enzymes in glioblastoma<sup>[41]</sup>. Looking at studies focusing on the expression patterns of HDACs in glioblastoma, these tissues seem to exhibit slightly and variably increased HDAC1, HDAC3, and HDAC6 expression levels as compared to non-neoplastic brain tissues examining both protein and mRNA within tissue samples<sup>[42]</sup>. The findings were further confirmed and even expanded to demonstrate that HDAC1 and HDAC3 expression levels correlated with WHO tumor grades, with the highest expression occurring in the most malignant gliomas. HDAC3, in particular, was correlated with poor survival. Another study observed that HDAC9 was overexpressed in glioblastomas with a poor prognosis<sup>[43]</sup>. The role of SIRT in glioblastoma is currently under debate due to equivocal findings across multiple studies. While many studies have correlated the down regulation of SIRT1 and SIRT6 in glioblastoma<sup>[44,45]</sup>, other studies have shown conflicting evidence as to whether the class II HDAC enzymes act as tumor suppressors or oncogenes<sup>[46,47]</sup>. The debate as to the role of class II HDACs will undoubtedly continue as the research into their roles becomes increasingly robust throughout the years. The classical HDAC family of enzymes is more clinically relevant as therapeutic agents have been developed to inhibit these aberrant enzymes. These therapeutic agents are currently undergoing clinical trials and are showing promise as potential new therapeutic modality for glioblastoma.

Another way to examine the HDAC expression in glioblastoma is to examine the effects and response displayed by these tumors when treated with HDAC inhibitors. Although this might be a more retrospective method of analysis and may be less clear due to the ambiguous mechanism of action of many HDAC inhibitors, this method may give some idea to which HDAC enzymes are aberrantly expressed in these tumors. We may be able to analyze the HDAC expression in tumor samples but the response of the tumor to a HDAC inhibitor as a potential therapy is a much more fruitful line of inquiry, emphasizing clinical results over cytologic curiosities. Ultimately, cytologic examination of glioblastoma tissue indicates which HDAC enzymes are aberrantly expressed, which goes on to inform which HDAC inhibitor may be useful for that tumor in particular, offering a potentially personalized approach to glioblastoma treatment. This review article will reveal that the answers, however, are not always clear-cut and highlight the complicated nature of these tumors, their protein expression, and their dysregulation leading to increased cell proliferation and malignant expansion.

## HDAC INHIBITORS

While biochemical investigation into HDAC enzyme activities was blossoming in the early 1970s, it was discovered in 1977 that millimolar concentrations of n-butyrate caused accumulation of acetylated histones<sup>[48]</sup>. It was subsequently confirmed that n-butyrate acted to inhibit histone deacetylation<sup>[49]</sup>. However, a direct causal relationship between these acetylated histones and n-butyrate was non-specific and unable to be verified, due to the documented effect of n-butyrate on cell membranes and many other enzymes other than HDAC. Later, the naturally occurring antifungal antibiotic trichostatin A (TSA) was discovered to be more potent for HDAC inhibition<sup>[50]</sup>. TSA, a hydroxamic acid compound, was found to inhibit cell cycle



progression through direct inhibition of HDAC enzymes, thereby providing genetic evidence of a direct cellular target that TSA acted to inhibit fungal growth. A few years later, a fungal cyclic peptide known as trapoxin was also found to strongly inhibit HDACs, this time displaying an irreversible enzymatic inhibition<sup>[51]</sup>. These compounds served as a proof of premise, where HDACs could be inhibited with the use of exogenous compounds. However, these compounds had yet to find a clinical use.

In 1998, two later compounds to be clinically significant HDAC inhibitors were reported in the literature: suberanilohydroxamic acid (SAHA) also known as vorinostat and FK228 also known as romidepsin<sup>[52,53]</sup>. Phase I clinical trials of FK228 conducted at the National Cancer Institute confirmed that this compound was effective for the therapy of cutaneous and peripheral T-cell lymphoma. This finding stimulated the interest of many researchers and began increased development of HDAC inhibitors towards the treatment of multiple cancers. After years of drug development, SAHA (vorinostat) was the first HDAC inhibitor approved for use in cancer chemotherapy<sup>[54]</sup> with FK228 following closely behind a few years later for approval in 2009. Multiple derivatives and novel compounds followed these two prototypic HDAC inhibitors, ultimately going on to have many investigational compounds being researched, all towards modifying the epigenetic expression in tumor cells through the inhibition of HDAC enzymes.

The HDAC inhibitors available today have wide variations in their function, structure, and mechanism. These inhibitors (similarly to their HDAC enzyme targets) can be divided into four classes on the basis of their chemical structure: hydroxamate, short-chain fatty acid (carboxylate), benzamide, and cyclic peptides [Table 2]. Adapted from recent investigations<sup>[55,56]</sup> and clinical trial records from the National Institutes of Health, these agents and their various progress towards approval by the United States Food and Drug Administration (FDA) for use in glioblastoma has been compiled. The hydroxamic acid derivatives now include the compounds of azlaic bishydroxamic acid, m-carboxycinnamic bishydroxamic acid, dacinostat (LAQ824), a novel HDAC inhibitor known only as AR-42, panobinostat (LBH-589), quisinostat, and suberic bishydroxamic acid, among the already known compounds TSA and SAHA. Short-chain fatty acid derivatives include pivaloyloxymethyl butyrate (pivanex, AN-9), sodium butyrate, buphenyl (sodium phenylbutyrate), and valproic acid. Benzamides include the lone HDAC inhibitor entinostat (MS-275) and cyclic peptides still include the lone inhibitor of romidepsin. Miscellaneous agents displaying HDAC inhibitory activity include diallyl trisulfide (DATS) and tubacin. The above agents have shown clinical efficacy against many clinical entities but are most notable in their ability to be used in cancer chemotherapy.

The precise mechanism for which HDAC inhibitors ultimately cause an anti-cancer effect is not completely understood. These agents typically inhibit cancer cell proliferation through causation of cell cycle arrest, differentiation, and/or apoptosis. Studies show that all HDAC inhibitors activate either the extrinsic or intrinsic pathways of apoptosis in cancer models (when used in a combination therapy), with some activating both apoptotic pathways<sup>[57]</sup>. As we will discuss later, these agents have also been found to play an immunomodulatory role against tumor cells as well. Ultimately, the mechanism for which these HDAC inhibitors exert their cellular changes does not need to be completely understood to observe clinical changes and the promise of these novel therapies. Some of these agents have already been approved for use and are in multiple phases of clinical trials towards the treatment of many pathologies [Table 2]. However, none of these agents have yet been approved for clinical use in the treatment of glioblastoma, a tumor that is in desperate need of novel therapeutics due to its dismal 5-year survival rates.

## HDAC INHIBITORS FOR ANTITUMOR EFFECTS IN GLIOBLASTOMA

Glioblastoma, as one of the deadliest human neoplasms with few effective treatment options, has frequently been a target of new treatment modality through clinical trials. HDAC inhibitors are no exception to this and these inhibitors have undergone multiple clinical trials to test their efficacy in glioblastoma. These agents have displayed both pre-clinical efficacy in their use, as well as efficacy in clinical use either as monotherapy

**Table 2. Histone deacetylase enzyme inhibitor classes**

HDAC inhibitor class	HDAC inhibitor(s)*	HDAC target	Clinical trial in GBM	Clinical trial for other uses
Hydroxamic acid	ABHA	HDAC classes I, II, and IV	Panobinostat in Phase II Belinostat in Phase II SAHA in Phase III	AR-42 in Phase I (acute myeloid leukemia)
	m-Carboxycinnamic CBHA			Panobinostat in Phase III (several cancers)
	LAQ824			Quinostat in Phase II (T-cell lymphoma)
	AR-42			Vorinostat in Phase III (cutaneous T-cell lymphoma and other cancers)
	Panobinostat			Belinostat indicated for use in treatment of peripheral T-cell lymphoma
	Quisinostat			
	SBHA			
	TSA			
	Vorinostat			
	Belinostat			
Short-chain fatty acid	Pivanex	HDAC classes I and II	Buphenyl in Phase II Valproate in Phase II	Pivanex in Phase II (non-small cell lung cancer)
	Sodium butyrate			Sodium butyrate in Phase II (endogenous antibiotics in gut)
	Buphenyl			Buphenyl indicated for use in treatment of urea cycle disorders
	Valproate			Valproate indicated for use in treatment of epilepsy, anorexia nervosa, panic attack, and anxiety disorders.
Benzamide	Entinostat	HDAC1, HDAC2, and HDAC3	Not available	Entinostat in Phase III (breast cancer)
Cyclic peptide	Romidepsin	HDAC1, HDAC2, HDAC3, and HDAC8	Phase I/II	Romidepsin indicated for use in treatment of cutaneous T-cell lymphoma and in Phase trials for many other cancers
Other	DATS	Unknown for DATS HDAC6 for Tubacin	Not available	Not available
	Tubacin			

\*HDAC inhibitors have been divided into four classes based on chemical makeup and HDAC classes they inhibit. Hydroxamic acid derivatives are some of the most well-described HDAC inhibitors and inhibit the classical HDAC family of enzymes. Panobinostat, belinostat, and SAHA are all at the clinical trial phase of development for use in GBM, with numerous other compounds showing efficacy in clinical trials for other tumors. Short-chain fatty acid HDAC inhibitors are also relatively well described and inhibit class I and II HDAC enzymes. Buphenyl and valproate are both in the clinical trials for use in GBM with numerous other compounds showing efficacy in clinical trials for other tumors. Entinostat is the sole benzamide derivative HDAC inhibitor and it has been shown to inhibit class I HDAC enzymes. This compound has not yet been used in clinical trials for treatment of GBM but has gone to a phase III clinical trial for treatment of breast cancer. Romidepsin is the sole cyclic peptide derivative HDAC inhibitor and it has also been shown to inhibit class I HDAC enzymes. This compound has gone to phase I and II clinical trials for use in GBM and it has been approved for treatment of cutaneous T-cell lymphoma. Finally, DATS and tubacin are miscellaneous HDAC inhibitors that are currently under investigation and they have variable effects on specific HDAC enzymes. HDAC: histone deacetylase; ABHA: azelaic bishydroxamic acid; CBHA: carboxycinnamic bishydroxamic acid; SBHA: suberic bishydroxamic acid; TSA: trichostatin A; DATS: diallyl trisulfide; GBM: glioblastoma multiforme

or in combination regimens<sup>[55]</sup>. Ultimately, there is a more vested interest in the clinical outcomes and efficacy, but in order for these clinical trials to be well reasoned there must be a strong research base and rationale behind the use of HDAC inhibitors.

There is a two-fold rationale for the use of HDAC inhibitors in glioblastoma therapy. First, HDAC inhibitors promote a more open chromatin conformation in the tumor cells and thereby permit the DNA alkylating chemotherapeutic agents (e.g., TMZ) to access genomic DNA and increase the sensitivity of the tumor cells for these agents. Second, HDAC inhibitors help reverse some of the abnormal genetic silencing in glioblastoma, where it is presumed that this will lead to enhanced cell-cycle arrest and apoptosis from the action of DNA damaging agents<sup>[58]</sup>. SAHA plays a unique role as an HDAC inhibitor that acts as a pan-inhibitor of all HDAC enzymes, while other HDAC inhibitors are more specific in their action. All the HDAC inhibitors, however, seem to cause increases in acetylation in histone and non-histone proteins and reactivate p21Waf1/Cip1, a protein that contributes to cell-cycle arrest due to its role as a tumor suppressor protein<sup>[59]</sup>. Traditionally, it has been believed that all HDAC inhibitors have difficulty in penetrating the BBB at low doses and require high doses for therapeutic effects. Some selective HDAC inhibitor classes such as the fatty acids<sup>[60]</sup> and benzamide compounds<sup>[61]</sup>, however, have shown increased penetration into the BBB on imaging studies. Interestingly enough, it also seems that there is some selectivity between HDAC inhibitors affecting tumor cells *vs.* normal cells. One older study, in particular, found that the antitumor effects of

hydroxamate-containing HDAC inhibitors displayed antitumor selectivity and did not affect somatic cells<sup>[62]</sup>, apprising the possibility of a safe agent with few toxicities to normal cells. Additionally, HDAC transcripts have been observed to be both increased and decreased in tumor cells undergoing exposure to HDAC inhibiting agents<sup>[63]</sup>. The results showed a lack of clear-cut cell cycle arrest effect, which the researchers recognized during other pre-clinical studies. The lack of specificity on HDAC substrates by HDAC inhibitors presents a mechanistic grey area concerning the use of HDAC inhibitors in glioblastoma specifically.

HDAC inhibitors have also shown efficacy in the preclinical arena towards the chemotherapy of GSCs. Targeting GSCs in particular is a major therapeutic undertaking as these cells often form the seeds of recurrence for glioblastoma after initial therapy and also confer resistance to previously used standard-of-care therapeutic agents. One study showed that the HDAC inhibitors TSA and valproic acid significantly reduced proliferation rates of GSCs by decreasing the amount of neural and embryonic stem cell surface markers expressed by these cells, indicating that these HDAC inhibitors stimulated differentiation in GSCs<sup>[64]</sup>. The HDAC inhibitor SAHA also demonstrated capabilities of slowing down tumor proliferation and triggering autophagy in GSCs, rather than induction of differentiation seen with TSA and valproic acid<sup>[65]</sup>. HDAC inhibitors have also been implicated for use in combination therapies against GSCs. Another study demonstrated that combination of the HDAC inhibitors SAHA, valproic acid, and sodium phenylbutyrate when used in combination with the FDA-approved proteasome inhibitor bortezomib caused high cytotoxicity against GSCs in cultures<sup>[66]</sup>. Specific chemotherapy that targets GSCs is in high demand as effective treatments for recurrent glioblastoma shows very poor efficacy. At least in the preclinical arena, HDAC inhibitors have demonstrated their efficacy in targeting GSCs in particular either through monotherapy or in combination with other known therapies.

Regarding current clinical trials under way for each specific HDAC inhibitor towards the treatment of glioblastoma, many HDAC inhibitors have shown considerable clinical promise but have yet to be approved by the FDA. These agents are said to be in the pre-clinical phase, where there are multiple rationales for specific inhibitors. Beginning with the examination of the hydroxamate derivative compounds, SAHA (vorinostat) has been shown *in vitro* to inhibit cell proliferation in glioblastoma cell lines independent of their p53 status, leading to an accumulation of cells arrested in the G2/M phase of the cell cycle, increased expression of anti-proliferative genes, and decreased levels of pro-growth genes<sup>[67]</sup>. SAHA additionally induces differentiation, apoptosis, and autophagy in human glioblastoma cell lines. As mentioned earlier, TSA is another hydroxamate compound akin to SAHA in HDAC targets. Similar to SAHA, TSA also induces differentiation and apoptosis in human glioblastoma cell lines, resulting in a higher expression of astrocyte-specific markers [i.e., glial fibrillary acidic protein (GFAP)] and reduced expression of vimentin and nestin (common markers of neuro-epithelial stem cells)<sup>[68]</sup>, increasing the recognizability of the tumor cells to the immune system. Of the short-chain fatty acid HDAC inhibitor class, valproic acid has been found to exhibit its antineoplastic effects through decreasing the activity and expression levels of matrix metalloproteinases (MMPs) in addition to the inhibition of activity of HDAC class I and II, thereby decreasing the invasiveness of glioblastoma cell lines<sup>[69]</sup>. Phenylbutyrate, another short-chain fatty acid HDAC inhibitor, has demonstrated its efficacy (like TSA) through increasing the expression of GFAP in human glioblastoma cells in culture as well as redistributing intracellular GFAP thereby enhancing gap junction communication between tumor cells through upregulation of the protein connexin 43<sup>[68,70]</sup>. Entinostat, the lone benzamide HDAC inhibitor, has been shown as a promising compound in the treatment of glioblastoma through its ability to significantly reduce cell growth, upregulate the cell cycle inhibitor p21Waf1/Cip1 and induce cell cycle arrest in the G0/G1 phase, and induce apoptotic cell death in glioblastoma cell lines<sup>[71]</sup>. Entinostat has also been shown to have some immunomodulatory roles similar to TSA through regulation of production of cytokines and inhibiting Treg cells in certain cancer models<sup>[72]</sup>. Romidepsin, the lone cyclic peptide HDAC inhibitor, has been shown at nanomolar levels in glioblastoma cell lines to cause inhibition of cell proliferation and induction of apoptosis (through the increased expression of the cell cycle inhibitor

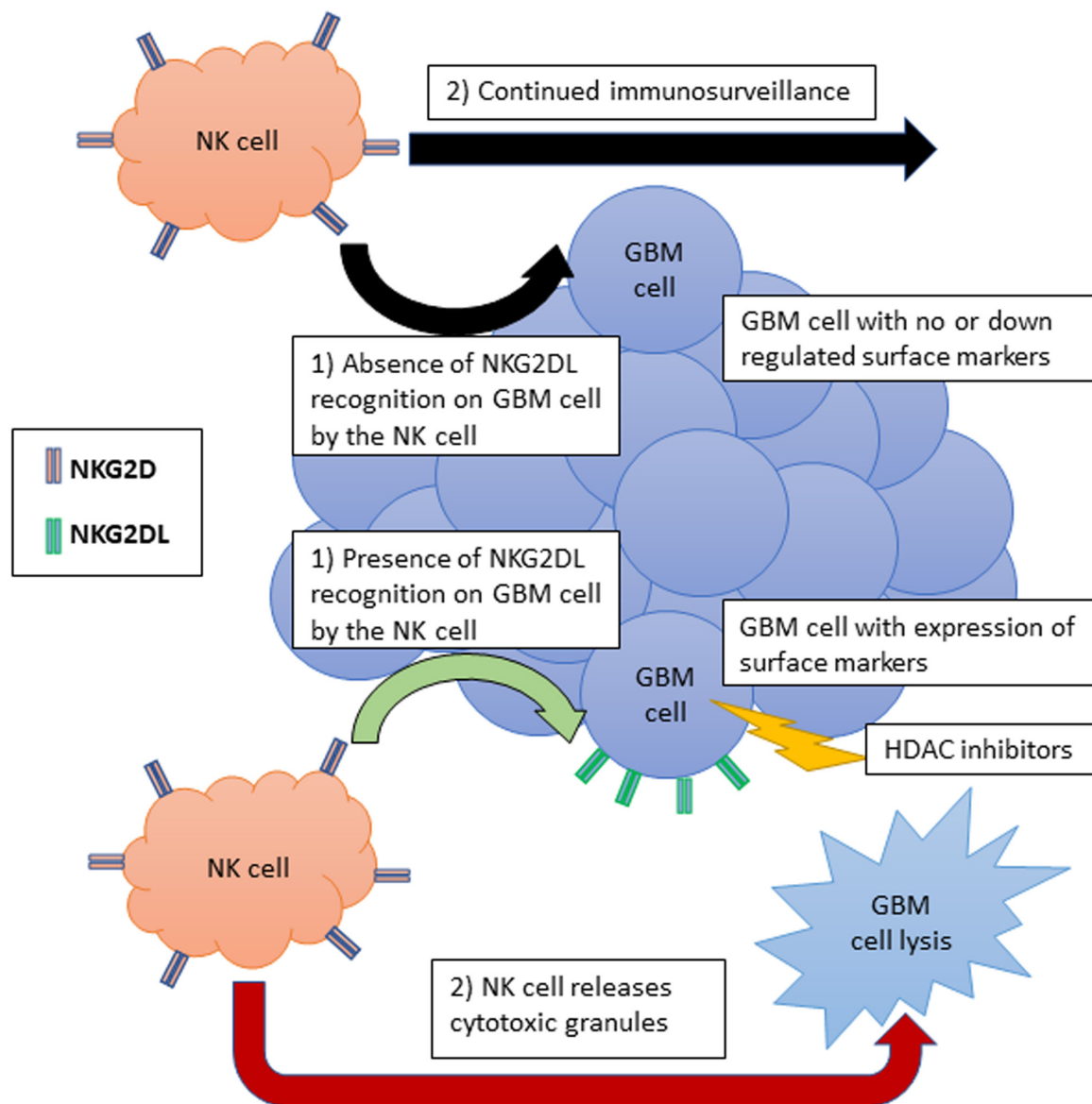
p21Waf1/Cip1 and the pro-apoptotic protein Bad and the decreased expression of the anti-apoptotic proteins Bcl-xL and Bcl-2)<sup>[73]</sup>. Finally, of the two miscellaneous HDAC inhibitors, DATS has been shown to cause upregulation of the cell cycle inhibitor p21Waf1/Cip1 and the tumor suppressor p53 in order to cause cell cycle arrest in glioblastoma cells and is unique in that it is derived from garlic and demonstrates less toxicity to normal cells than other HDAC inhibitors<sup>[74]</sup>. Tubacin, the other miscellaneous HDAC inhibitor, is a specific for HDAC6<sup>[75]</sup> and it has been proposed to be useful because HDAC6 is known to be increased in certain high-grade gliomas. All the above HDAC inhibitors have shown considerable promise for growth inhibition in glioblastoma, but only a few of these agents have made it into the clinical trials as of now.

Only a few selected HDAC inhibitors that have shown promise in the pre-clinical realm translate so seamlessly over to show efficacy in the clinical realm. Vorinostat, romidepsin, and valproic acid are particularly notable to have seen translational promise in the preclinical realm as well as in the clinical realm. Vorinostat as a monotherapy progressed through multiple Phase I and Phase II trials, with the results of one Phase II trial indicating that it was well tolerated in recurrent glioblastoma patients, and its efficacy was seen to extend life by a few months in a subpopulation of those with recurrent glioblastoma<sup>[76]</sup>. Romidepsin also went through both Phase I and Phase II trials but had disappointing outcomes in progression free survival with the conclusion that although the drug demonstrated success in the preclinical arena, when used in clinics an inadequate amount of the drug reached the actual tumor in the CNS<sup>[77]</sup>. However, this agent showed success when used in combination therapies. Valproic acid similarly showed success in clinical trials, but only when used in combination therapies and not when used as a monotherapy<sup>[78]</sup>. In fact, many other HDAC inhibitors listed in Table 2 in various trials for use in glioblastoma are in combination therapies and may yet show results when combined with the standard-of-care agents. However, HDAC inhibitors when used as monotherapy have yet to yield the progression free survival results that the preclinical mechanistic evidence would suggest, with an exception to vorinostat (and even then, only modestly so). To understand the full picture of these promising new agents, one must look at both the preclinical and the clinical data housed in trials. Unfortunately, there seems to be a wealth of new mechanisms to be revealed and understood as to the biological pathways these agents are inhibiting. A more profound understanding of glioblastoma pathogenesis and the associated aberrant pathways inhibited by these agents is essential to translate the benefits from the preclinical bench to the clinical arena.

## HDAC INHIBITORS FOR ENHANCING ANTITUMOR IMMUNITY IN GLIOBLASTOMA

While there have been a variety of preclinical studies regarding the effects of HDAC inhibitors specifically in glioblastoma, one of the most interesting effects is alteration of the tumor itself to increase tumor susceptibility to antitumor immune attack. Many cells of the immune system act as surveillance cells, effectively patrolling the body to eliminate neoplastic cells as soon as they are found<sup>[79]</sup>. However, many tumors are notorious for down regulating these markers on their surfaces, effectively “hiding” from the immune system to evade elimination and continue their unfettered growth. While there are many important cell types (microglia, T cells, etc.) involved in the surveillance of the body’s somatic tissues for signs of pathological changes, one of the cell types most important to the preclinical mechanism of HDAC inhibitors and antitumor immunity are NK cells. These cells act to “check” or surveil surface proteins displayed on the exterior of many cells, checking these cells for signs of stress, infection, or neoplastic change. If an NK cell finds a cell within the body that has undergone one of these pathological changes, the NK cell releases cytotoxic granules containing toxic compounds known as perforins and granzymes, which act synergistically to induce apoptosis in the target cell. These NK cells have been described in the literature to be the agents that lyse GBM cells as they are recognized during their surveillance, with HDAC inhibitors playing a role in upregulating the surface markers that help to mark these malignant cells as a target for elimination<sup>[19,22]</sup>.

The NK cells possess a constitutively expressed receptor on their surface known as natural killer group 2D (NKG2D) that is essential for recognition of abnormal human tissues<sup>[80]</sup>. This receptor recognizes a ligand



**Figure 2.** Natural killer (NK) cell antitumor immunity under histone deacetylase (HDAC) inhibitor influence. A tumor such as glioblastoma multiforme (GBM) is able to eschew immune surveillance by NK cells through either down regulation of surface marker [i.e., natural killer group 2D ligand (NKG2DL)] or through the activation of matrix metalloproteinases to degrade surface marker once they reach the tumor cell's surface. Some selected HDAC inhibitors such as trichostatin A have been shown to upregulate surface markers in GBM. This upregulation of surface markers on the tumor cell's surface makes the tumor able to be recognized by the immune system (through binding of natural killer group 2D to NKG2DL), causing the NK cells to release cytotoxic granules and leading to apoptosis in the GBM cell

known as natural killer group 2D ligand (NKG2DL) that is expressed by somatic cells in times of stress, marking them for destruction via release of cytotoxic granules from NK cells<sup>[81]</sup>. However, GBM cells have found a mechanism for evading this natural antitumor immunity through the down regulation of NKG2DL, thereby avoiding surveillance, or through the activation of MMPs that act to shed the natural expression of these ligands and release them into the microenvironment surrounding the tumor<sup>[82]</sup>. Interestingly enough, it is now known that expression of this specific ligand in GBM cells is regulated by HDAC enzymes, where overexpression of these enzymes in tumor cells is effectively silencing the genes responsible for the expression of these surface markers [Figure 2]. HDAC inhibitors have therefore been shown to induce the expression of these ligands on the surface of GBM cells, thereby allowing these cells to be recognized by the immune system and subsequently be destroyed<sup>[83]</sup>.



Other leukocytes that have been implicated in antitumor immunity among NK cells also include Treg cells and microglia. Instead of priming the tumor cells for removal by the immune system, current inquiry has looked into the role of these leukocytes in the tumor microenvironment, and how their inhibition may increase the tumor's susceptibility to clearance by the immune system<sup>[84]</sup>. One pilot study in particular looked at lymphodepletion of Treg cells through the use of monoclonal antibodies in those with glioblastoma and showed enhanced antitumor immunity, as it had been shown in the past that these Treg cells were associated with immunosuppression of glioblastoma<sup>[85]</sup>. Depleting the Treg cells through the use of the anti-CD25 monoclonal antibody daclizumab was able to paradoxically enhance antitumor immunity. Additionally, another study looked at using anti-PD-1 and anti-CTLA-4 antibodies for the use of inhibiting Treg cell function as well, which showed improved survival in mouse models<sup>[86]</sup>. Microglia have been similarly targeted to enhance antitumor immunity, as they have increased presence within the GBM microenvironment and are assumed to have roles in local immunosuppression. Inhibition of the signal transducer and activator of transcription 3 (STAT3) pathway within tumor cells has shown improved outcomes in mouse models specifically, with one study using the siRNA-based method to activate these cells within the tumor microenvironment and subsequently slow tumor growth<sup>[87]</sup>. Another study showed success using the same rationale but utilizing the miR-124 inhibition of the STAT3 pathway to enhance antitumor immunity<sup>[88]</sup>. While these studies have demonstrated promising concepts for future investigation regarding antitumor immunity in leukocytes, these effects are largely limited to the tumor microenvironment and the biggest successes have only been demonstrated in mouse models or had a very small sample size. Additional investigation is obviously required before these potential therapeutic modalities are ready for human trials.

Of the known HDAC inhibitors, TSA seems to show promise in the preclinical realm for enhancing antitumor immunity; but unfortunately, when brought into the clinical arena, TSA showed high toxicity and low efficacy. While this compound has been shown to upregulate NKG2DL expression on GBM cells directly, it is unclear whether this action is due to epigenetic transcriptional alteration within the tumor cell or this is due to reduction of secretion of MMPs<sup>[69]</sup>. The immunostimulatory effect of TSA was shown to be also dependent upon the presence of NK cells, as evident from the use of an anti-NKG2D antibody significantly reducing the amount of observed GBM cell lysis *in vitro*. While this compound showed considerable preclinical promise, its high toxicity and low efficacy has made other HDAC inhibitors such as vorinostat, romidepsin, and valproic acid as more promising candidates for potential future monotherapy in GBM. These HDAC inhibitors unfortunately do not display the same antitumor immunity as other HDAC inhibitors in the preclinical arena but are the most likely candidates to be used for future monotherapy or combination therapy in clinical trials.

While HDAC inhibitors have been used to treat cancers successfully in the past and have seen modest success in their use against GBM specifically, this is the first time that these agents have been utilized as an immunotherapy regimen in GBM. As it has already been described in this article, while an agent may see mechanistic success in the laboratory setting this may or may not translate to the clinical realm through the process of FDA approval and clinical trials. Studies such as these offer exciting possibility of new therapeutic modalities for a formidable clinical challenge that is in desperate need of innovation.

## IMMUNOTHERAPY IN CONTROLLING GROWTH OF GLIOBLASTOMA

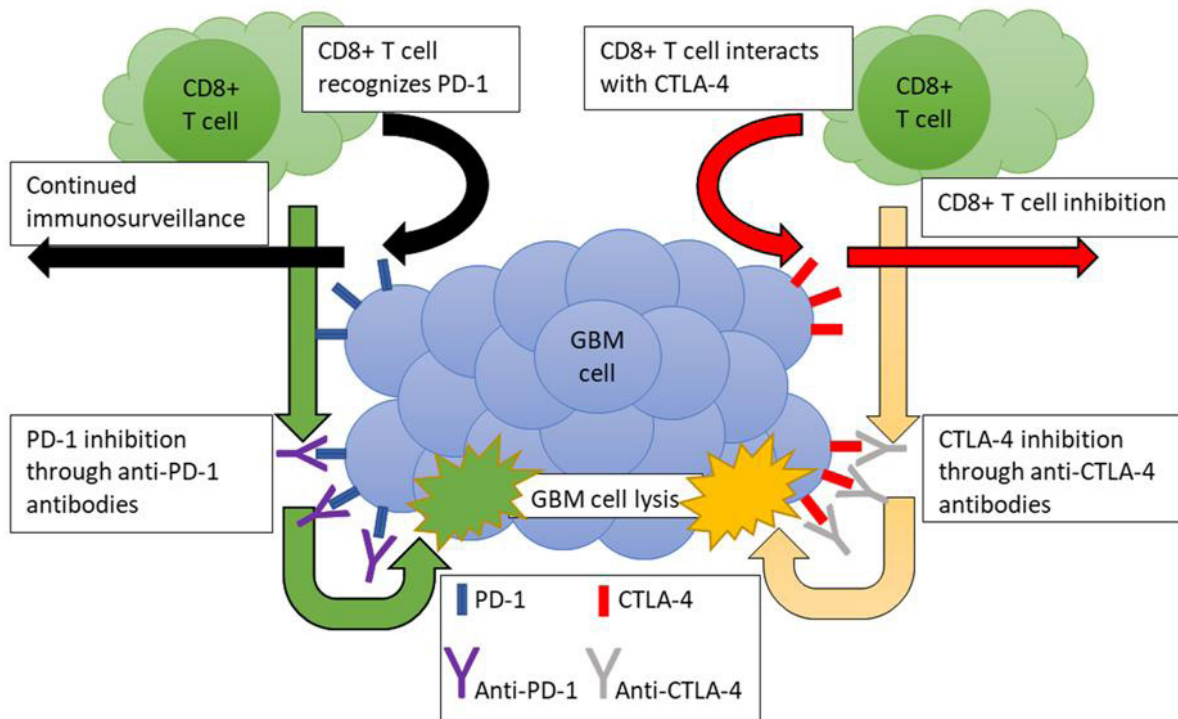
One of the most exciting new therapy modalities being examined for the treatment of glioblastoma is immunotherapy and immunomodulation, or harnessing/modifying the body's immune system to help fight the tumor directly. However, while in theory these therapies may be very promising, in practice the tumors themselves have multiple mechanisms of immunosuppression that lead to promising *in vitro* results, but further studies do not necessarily see the same *in vivo* or clinical results<sup>[89]</sup>. These tumors cause systemic immunosuppression through their release of cytokines, which inhibit lymphocyte proliferation and promote

production of the well-characterized immunosuppressive factors such as transforming growth factor- $\beta$ , interleukin-10, prostaglandin E2, and gangliosides<sup>[90]</sup>. These tumors also secrete the chemoattractants such as monocyte chemoattractant protein-1, colony stimulating factor-1, granulocyte/macrophage colony stimulating factor-1, and hepatocyte growth factor to recruit microglia to the local tumor microenvironment in order to support tumor cell proliferation and tumor growth, as well as secrete factors that lead to local immunosuppression and inhibition of the remaining immune system cells that are now unavailable to attack this tumor<sup>[43]</sup>. Finally, these tumors also have immunosuppressive antigens on their cell membrane surfaces and secrete factors that lead to further inhibition of the immune system from properly attacking these tumors<sup>[91]</sup>. With these mechanisms in place in glioblastomas, it becomes essential to first understand the immunosuppressive mechanisms employed by these tumors before delving into the immunotherapy/immunomodulation mechanisms that have been showing such preclinical promise and possibly to explain the lack of translation of this promise into the clinical realm.

Despite the immunosuppressive action inherent in glioblastoma, this tumor has been the subject of multiple studies using multiple immunomodulatory methods besides HDAC inhibitors. One of the more exciting strategies is the use of “trained” T cells directed towards known tumor antigens, also known as CAR-T cell therapy. This therapy modality has been applied towards glioblastoma, with mixed results for a variety of reasons. These barriers to successful therapy include the previously-discussed barriers to cellular delivery and immunosuppressive microenvironment as well as proper selection of appropriate glioblastoma antigens to train the T cells<sup>[10]</sup>. However, a recently published high-profile case study has shown regression of recurrent GBM following the use of this CAR-T cell therapy<sup>[92]</sup>, heralding this particular treatment modality as extraordinarily effective in certain cases and in certain tumor types. Ultimately, this treatment modality shows considerable promise and with initial Phase I trials suggesting that this therapy is safe without dose-limiting side effects, this strategy will be very likely to continue to be considered as our lists of GBM antigenic targets as well as continue to increase as our understanding of these tumors becomes more robust<sup>[93]</sup>.

Another immunomodulatory treatment modality that has shown promise in recent years is the use of immune checkpoint inhibitors, which are agents that help “unblock” the regulation induced by tumor cells on the immune system, priming the tumor cells for killing. Specific immune checkpoint proteins that have been investigated for immunotherapy of GBM include: PD-1/PD-L1, CTLA-4, T cell immunoglobulin and mucin containing protein-3, and indoleamine-(2,3)-dioxygenase<sup>[11]</sup>. The rationale behind these therapies involves the use of monoclonal antibodies designed to target these surface markers in order to increase the tumor’s susceptibility to immune attack by cytotoxic T cells [Figure 3]. These immune checkpoint proteins restrain immune responses and thereby prevent T cells from killing the tumor cells. When these proteins are gridlocked with monoclonal antibodies, the restraints on the immune responses are released and T cells turn into weapons to kill tumor cells. Specific to glioblastoma, these therapies have been explored as a promising crop of new therapeutic targets<sup>[94]</sup>. While these targets have shown promise in clinical trials, the ultimate assessment of these agents are mixed at best. Each of these agents has been speculated to be a useful therapeutic modality when combined with other chemotherapy, radiation, or with other immunomodulatory treatments<sup>[95]</sup>. Unfortunately, these strategies have yet to show the promising results in the clinical realm.

Finally, GBM is the target of yet another immunomodulatory treatment modality, the use of vaccine therapy to prime the immune system to fight the tumor directly and recognize recurrences, much in the same way our immune system already does with many infectious agents. These strategies have utilized multiple targets in an attempt to activate the immune system in a way where it is able to eradicate the tumor, which include: peptide vaccines, polyvalent dendritic cell vaccines, and heat shock protein vaccines. Again, akin to many other agents discussed in this article, these agents have shown mixed results depending on which clinical trial you examine and have been only suggested to supplement the already established standard-of-care treatments<sup>[96]</sup>. The movement for vaccine strategies for the treatment of GBM allows for considerable targeted



**Figure 3.** Potential immunotherapy for glioblastoma multiforme (GBM) using anti-programmed death-1 (PD-1) and anti-cytotoxic T lymphocyte associated antigen-4 (CTLA-4) antibodies. Anti-PD-1 and anti-CTLA-4 antibodies have been utilized in different human malignancies to promote antitumor immunity with enormous success in selective cases. This antitumor immunity is proposed to be mediated through disinhibition/stimulation of cytotoxic T cells for eliminating the malignant cells. Anti-PD-1 inhibits the interaction of programmed death-ligand 1 on cytotoxic T cells, making the T cells believe that the cell they are interacting with is foreign. Similarly, anti-CTLA-4 inhibits the interaction of the inhibitory CTLA-4 surface marker with B7 surface marker of the cytotoxic T cell, allowing for recognition of the malignancy by the T cells. The cytotoxic T cells then release their cytotoxic granules, leading to apoptosis in the GBM cells

therapy opportunities, with examination of personal tumor elements and vaccines that have been shown to effectively combat those tumors in the clinical arena from past studies as well in combination with existing standard-of-care regimens<sup>[97,98]</sup>.

Vaccines will continue to evolve as our understanding of tumor immunology continues to evolve, which is the crux of a comment on the progress in this certain field. Our understanding of tumor immunology is quickly expanding, and we are bringing into relief the degree of complexity in the tumor microenvironment. Still significant barriers to overcoming tumor-mediated immunosuppression, treatment delivery in the CNS, and proper selection of the correct targeted therapy are just a few of the limitations this therapy modality must overcome. However, a more profound mechanistic understanding of these tumors and more data regarding the efficacy of the immunotherapy treatment modality are showing promise. Perhaps immunotherapy for glioblastoma will become the panacea as it has been promised, despite the considerable work that must be undertaken and continued to reach such a horizon<sup>[99,100]</sup>.

## CONCLUSION

While glioblastoma continues to present a formidable preclinical challenge for researchers, further inquiry into the molecular pathogenesis, aberrant cellular pathways, and tumor immunology will ultimately aid in the development of more targeted therapies for a clinical entity that has yet to find a solution. Success in treating a disease with such a dismal survival rate will come from a well-rationalized approach that will translate into real-world clinical measures such as progression free survival. HDAC inhibitors are another promising treatment modality being investigated to combat this insidious malignancy. While these therapies

may show promise, the mechanistic minutiae of why a therapy may or may not be effective is just as valuable. Continued work is required in the field of glioblastoma, as the promise that has been shown by these agents is begging to be brought to its fullest potential and may yet offer hope to those diagnosed with an illness long surrounded by pessimism, dread, and anxiety.

## DECLARATIONS

### Authors' contributions

Conceptualized the theme and conducted the literature review process: Yelton CJ

Preparation of the manuscript, interpretation of subtopics, preparation figures, approval of the final version to be published: Yelton CJ, Ray SK

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Review

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# Immunotherapy and checkpoint inhibitors for gliomas

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## Abstract

Glioma treatments are faced with challenges, including the inability to fully eliminate cancer stem cells, the immunosuppressive tumor microenvironment, and the blood brain barrier. Although progress has been made with surgical, radiation, and chemotherapies, prognosis for patients remains poor. Rapidly emerging immunotherapies may be able to address the challenges that conventional techniques cannot. Immunotherapies manipulate the patient's immune system to selectively combat malignancies. Therapies often work to enhance T-cell and natural killer (NK) cell function, which can both eliminate tumor cells and enhance remission. Vaccines encourage *in vivo* development of anti-tumor T-cells and NK cells, while adoptive transfer techniques focus on engineering immune cells *ex vivo* before reintroducing them to patients. Vaccine and adoptive transfer therapies have been shown to induce enhanced immune responses in patients but have not always correlated with improved outcomes, likely because of the tumor immunosuppressive microenvironment. Checkpoint inhibitors can impair these tumor immunosuppressive capabilities. Although no one treatment has been able to consistently eliminate gliomas and maintain remission, combinations of vaccines or adoptive transfer techniques in conjunction with immune checkpoint inhibitors offers promise.

**Keywords:** Glioma, immunotherapy, checkpoint inhibitors, vaccines, T-cells, dendritic cells



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## INTRODUCTION

Gliomas arise from different glial cell populations and can manifest as astrocytic tumors (astrocytoma, anaplastic astrocytoma, and glioblastoma), oligodendrogliomas, ependymomas, and mixed gliomas. Glioblastoma multiforme (GBM) is the most malignant, invasive, and common glioma, accounting for over 50% of all diagnosed brain tumors. A patient with GBM possesses poor prognosis, as typical survival is 14-15 months following diagnosis. Standard treatment includes surgical resection, radiation therapy, and chemotherapy [temozolomide (TMZ)], but these often result in limited success<sup>[1]</sup>. This is partially due to treatment limitations, including infiltration of GBM cells to surrounding brain tissue, intratumoral heterogeneity, and therapy passage across the blood brain barrier (BBB)<sup>[2]</sup>.

The central nervous system (CNS) has long been considered an immune-privileged site, evidenced by the presence of the BBB, lack of lymphatic vessels, and absence of major histocompatibility complex (MHC) - positive antigen presenting cells (APCs)<sup>[3]</sup>. However, recent work suggests that the CNS has a closer relationship with the peripheral immune system. Soluble antigens of the cerebrospinal fluid drain into the cervical lymph nodes, providing T cells activation before transport to the site of inflammation<sup>[4]</sup>. Leukocytes are known to gain partial access to the CNS through the choroid plexus, across the superficial leptomeningeal vessels, and into the perivascular space<sup>[5,6]</sup>. Brain tumor progression further compromises the integrity of the BBB, allowing these T cells access to the brain<sup>[7]</sup>. Given that immune cells can permeate the BBB while maintaining cancer cell-specific targeting, immunotherapies have begun to establish their promise in GBM treatment.

In this review, we will examine recent advances made in immunotherapies for GBM, focusing on harnessing apoptotic functions in natural killer (NK) cells, blocking checkpoint inhibitors to unmask malignancies, and inducing systemic response by vaccine administration to target tumors. We will also highlight clinical trials using these immunotherapies. Finally, we will address therapy challenges and discuss the need for further refinement in applications specific to brain tumors.

## T-CELLS

The ratio of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells is a prognostic marker in many malignancies and has been found to inversely correlate with progression-free survival and overall survival (OS) in GBM<sup>[8]</sup>, demonstrating an avenue for immunotherapy intervention.

T-cells maturing in the thymus may develop into CD4<sup>+</sup> or CD8<sup>+</sup> T-cells, also known as helper T-cells or cytotoxic T-cells, respectively. They are activated upon APC interaction. APCs recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors, such as toll-like receptors. Once PAMPs are recognized before or during endocytosis, antigens, usually peptides, are loaded onto MHC-I or MHC-II proteins. Antigens that are loaded onto MHC-II proteins activate CD4<sup>+</sup> T-cells, which further activate CD8<sup>+</sup> T-cell responses. This contrasts with antigens that are loaded onto MHC-I proteins, which activate only CD8<sup>+</sup> T-cells<sup>[9]</sup>.

Induction of helper T-cell differentiation from CD4<sup>+</sup> T-cells is orchestrated by cytokine stimulation<sup>[10]</sup>. Helper T-cells promote anti-tumor response by secreting pro-inflammatory cytokines interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF). These cytokines then activate death receptors on the tumor cell surface, consequently triggering dendritic cell (DC) cytotoxic functions. It is these functions that eradicate tumor cells. T regulatory cells (Tregs) often modulate the immune response through the secretion of anti-inflammatory cytokines tumor growth factor- $\beta$  and interleukin (IL)-10. While Treg cytokine secretion is aimed to prevent a dramatic immune response, Tregs may inadvertently contribute to tumor development by suppressing pro-inflammatory anti-tumor responses<sup>[11]</sup>.

CD4<sup>+</sup> and CD8<sup>+</sup> T-cells can further differentiate into effector or memory T-cells, which are essential in delivering a robust secondary immune response after re-exposure to antigen<sup>[10]</sup>. CD8<sup>+</sup> effector T-cells attack tumor cells by releasing perforin and granzyme cytotoxic molecules and producing pro-inflammatory cytokines TNF and IFN- $\gamma$ . During antigen clearance, most CD8<sup>+</sup> effector T-cells undergo apoptosis, with a small margin surviving. When high levels of antigens persist in an environment, these remaining CD8<sup>+</sup> T-cells progress into a state of T-cell exhaustion. This situation is commonly seen in cancers with solid tumors like GBM. In the T-cell exhaustive state, inhibitory receptors are overexpressed, cytokine signaling pathways are dysregulated, and altered metabolic fitness occurs<sup>[12]</sup>, leading to altered T-cell functioning and unrestrained tumor growth. Novel strategies and new approaches have been developed in attempts to circumvent these hurdles to immunotherapy for gliomas.

## ADOPTIVE T-CELL TRANSFER

Adoptive T-cell transfer (ACT) involves the collection and *ex vivo* expansion of autologous anti-tumor T lymphocytes. These cells are then reinfused into the patient, delivering a potent and focused response. This approach provides the immune system and tumor microenvironment with an already abundant, activated T-cell population that can proliferate *in vivo* to maintain antitumor functions. ACT therapy can provide benefits to immunocompromised patients as it eliminates the need for self-induced antigen presentation. This feature, along with the ability for T-cells to bypass the BBB, suggests that ACT may be particularly effective in brain tumor treatment<sup>[13]</sup>.

There are several avenues to the ACT approach, but the furthest advanced is the chimeric antigen receptor T-cell (CAR T) therapy. After isolating T-cells from the patient, these cells are genetically engineered to express receptors that mediate tumor cell destruction after reinfusion to patients. CAR T targeting of B cell marker CD19 has shown great efficacy in lymphoblastic leukemia and B cell lymphomas, but applications in the treatment of solid tumors have only begun to be explored<sup>[14,15]</sup>. Progress has been made in CAR T treatments for brain tumor targeting, particularly utilizing epidermal growth factor receptor (EGFR)vIII [Table 1]. Given that EGFRvIII is known to be prevalent in gliomas, CAR was directed against EGFRvIII and used in a phase I clinical trial for recurrent GBM<sup>[16]</sup>. All patients given CAR T EGFRvIII intravenous infusions exhibited decreased expression of EGFRvIII in tumors, indicative of CAR on-target effects. Flow cytometric analysis of CD3<sup>+</sup> T-cells detected engraftment of CAR T-EGFRvIII cells in the peripheral blood. These findings demonstrated CAR T transient growth advantage as compared to endogenous lymphocytes, and that rescue of normal T-cell activity can be achieved. This is especially applicable to immunocompromised patients previously administered doses of TMZ and radiation. None of the infused subjects presented symptoms of tumor toxicity nor cytokine release syndrome, and there was no cross-reactivity of wild type EGFR<sup>[17]</sup>.

Other clinical trials have shown that through administration of glioma associated antigen IL13R $\alpha$ 2 in CAR T-cells, patients with recurrent GBM showed transient, anti-tumor responses. In metastatic GBM, significant tumor regression could be observed after 4-1BB co-stimulation and a mutated IgG4-Fc linker was incorporated into administered CAR T-cells which enhance antitumor potency and reduced off target interactions. While both intracavitary and intraventricular administration of CAR T populations was performed, intraventricular administration was found to achieve regression of all CNS tumors<sup>[18]</sup>. These preliminary findings illustrate the potential for CAR T mass manufacture, designed to address function and toxicity deficiencies.

Challenges in transitioning ACT to the clinic include the risk of inducing graft vs. host disease, which may arise through allogeneic T-cell transfusion. Identification of toxicity prior to human administration is another challenge, largely due to the lack of representative immune systems in pre-clinical models. Introduction of a robust T-cell population carries its drawbacks. Off-targeting can present as cross-reactivity of the T-cell receptor with an antigen that possesses similar structure to the target antigen. This can result in



**Table 1. Clinical trials using adoptive T-cell transfers to treat gliomas**

Identifier	Trial name	Treatment	Phase	Diagnosis (newly diagnosed or reoccurring)
NCT02844062	Pilot study of autologous anti-EGFRvIII CAR T-cells in recurrent GBM	Autologous anti-EGFRvIII CAR T	I	Reoccurring
NCT03283631	Intracerebral EGFRvIII CAR-T-cells for recurrent GBM (intercept)	Autologous anti-EGFRvIII CAR T	I	Reoccurring
NCT03389230	Memory-enriched T-cells in treating patients with recurrent or refractory grade III-IV glioma	Leukapheresis, autologous HER2(EQ)BBζ/CD19t+	I	Reoccurring
NCT03423992	Personalized CAR T immunotherapy for patients with recurrent malignant gliomas	Autologous anti-EGFRvIII, IL13Rα2, HER2, CD133, EphA2, GD2 CAR T	I	Reoccurring
NCT02208362	Genetically modified T-cells in treating patients with recurrent or refractory malignant glioma	IL13Rα2-specific, hinge-optimized, 41BB-costimulatory CAR/truncated CD19-expressing autologous T lymphocytes	I	Reoccurring
NCT02209376	Autologous T-cells redirected to EGFRvIII - with a chimeric antigen receptor in patients with EGFRvIII + GBM	CAR T-EGFRvIII T-cells	I	Patients with residual

EGFR: epidermal growth factor receptor; CAR T: chimeric antigen receptor T-cell; GBM: glioblastoma multiforme; HER2: human epidermal growth factor receptor 2

cytotoxic effects to otherwise healthy tissue<sup>[19]</sup>. Conversely, toxicities can also arise through on-target effects. This generally manifests as successful binding to the antigen expressed in environments not specific to the tumor<sup>[20]</sup>. Cytokine release syndrome is another condition that results from the release of mass quantities of cytokines, an indirect result of the release of mass quantities of T-cells<sup>[21]</sup>. However, downregulation in the prevalence of these cytokines can be achieved by receptor blockage. Specifically, in treatment focused on acute lymphoblastic leukemia, a patient administered tocilizumab (an IL-6 receptor inhibitor) was able to reverse cytokine storm syndrome symptoms yet still maintained the T-cell population and continued to derive benefits from ACT therapy<sup>[22]</sup>. Thus, while these side effects are serious, continued refinement protocols can be made so symptoms are less severe and more manageable.

Current efforts in ACT aim to target solid tumors and optimize gene transfer. Methods of achieving gene transduction to T lymphocytes include retroviral and lentiviral gene delivery. Positive efficacy in gene transduction may be observed, but integration of genes may prefer certain areas of the genome over others. Poor integration of genes could result in mutagenesis and overexpression or disruption of nearby genes in T-cells<sup>[23,24]</sup>. Additionally, patients' immune systems may react to the vectors themselves, and these genotoxic events may interfere with T-cell delivery. To circumvent these issues, engineering of CAR T-cells by piggyBac and Sleeping Beauty transposons has gained momentum<sup>[25,26]</sup>. These methods offer reduced manufacture cost, increased simplicity, and less good manufacturing practice requirements. Gene expression remains unperturbed and foreign proteins that could result in adverse effects are absent. Treatments utilizing Sleeping Beauty have just reached phase I clinical trials<sup>[27]</sup>. Optimization and precision of these transposon systems may prove crucial to improved ACT safety.

## NK CELLS

NK cells demonstrate potent anti-tumor immunity. Unlike T-cells, transfusions of NK cells are not complicated by graft-host-disease. NK cells detect and eliminate cell abnormalities and are found in lymphoid and non-lymphoid organs<sup>[28]</sup>. Activated NK cells secrete perforin and granzymes to induce apoptosis in target cells. Ligands like killer immunoglobulin-like receptors are expressed on healthy cells and can inhibit the destructive activity of NK cells. Antibodies, cytokines, natural cytotoxicity receptors and transmembrane protein NK2GD on infected or transformed cells provide activating signals to NK cells<sup>[29]</sup>. Conversely, downregulation of MHC-I in tumor cells depletes inhibitory signals to NK cells. It is these shifts between activating and inhibitory signals that allow NK cells to selectively target abnormal cells. This is the premise of anti-tumor NK cell immunotherapies<sup>[30]</sup>.

Studies aimed at heightening NK cell activity for immunotherapies have done so through genetic engineering or by stimulation of NK cells directly *in vivo* or *ex vivo* [Table 2]. The tumor microenvironment is known to be immunosuppressive which correlates with reduced NK cell activity<sup>[31]</sup>. Because of this, few NK cells achieve activation to carry out NK cell-mediated lysis. By stimulating NK cells, however, the immunosuppressive tumor microenvironment may be overcome. Efforts to stimulate NK cell proliferation and activity have focused on exposure of NK cells to cytokines including IL-2, 12, 15, 18 and 21. Once stimulated, NK cells become lymphokine-activated killer cells. These cells have demonstrated increased levels of cytotoxicity towards malignant tumors and proliferate at a greater rate<sup>[29]</sup>.

Clinically, NK cell stimulation with IL-2 was approved for treatment of metastatic renal cancer by the FDA in 1992. High-doses of IL-2 has demonstrated efficacy in treating various cancers, but increasing doses also increases the risk of severe adverse effects. A phase III trial comparing IL-2 doses for liver and bone metastases and primary tumors showed that the response rate for high-dose IL-2 was significantly higher than all other groups. It was concluded high-dose IL-2 were necessary for significant clinical benefits, despite the possible negative effects<sup>[32]</sup>.

IL-21 stimulates NK cells and CD8<sup>+</sup> T-cells while also increasing the production of IFN- $\gamma$ . Together with IL-2 and IL-15, IL-21 enhances cytotoxic effects of NK and CD8<sup>+</sup> T-cells<sup>[33]</sup>. A phase II clinical study treating melanoma patients with IL-21 found that antitumor efficacy of IL-21 is comparable to that of high-dose IL-2. The treatments were well tolerated among patients and resulted in few adverse actions<sup>[34]</sup>.

IL-15 has been tested in phase I trials to monitor the reactions among patients after administration. The cytokine was given as bolus intravenous infusions to patients with metastatic malignant melanoma and renal cell cancer. The treatments caused a large swing in the distribution of lymphocytes within the blood, suggesting its importance to the activation of NK cells and their cytotoxicity. Many adverse reactions were recorded, however, which is thought to be the result of the method of administration<sup>[35]</sup>.

IL-12 is another cytokine under investigation for use in immunotherapy. Studies in preclinical models using IL-12 have shown strong antitumor effects<sup>[36]</sup>. In one such study, the rejection of gliomas in mice was found to be significantly enhanced in those expressing IL-12 in the CNS, as compared to those without<sup>[37]</sup>. This gives evidence that the expression of IL-12 cytokine can be a major factor in anti-tumor response through stimulation of the immune system.

NK cell function is heavily dependent on cytokine support. But, even with administration of additional cytokines, the tumor microenvironment may limit NK cell activation. To overcome dependence on exogenous cytokines, genetically engineered NK cells have been explored for their ability to surpass the tumor microenvironment. One study examined whether transduced expression of a nonsecretory, membrane-bound form IL-15 (mbIL15) could sustain NK cells. The mbIL15 NK cells had enhanced survival and viability compared to mock-transduced NK cells and NK cells that expressed non-membrane bound IL-15. Because mbIL15 NK cells are less dependent on endogenous signaling molecules, their activity and cytotoxicity against solid tumors is resilient to immunosuppressant effects of the tumors<sup>[38]</sup>. Genetic engineering of NK cells to self-activate may prove more effective than stimulation from endogenous cytokines. Engineered NK cells avoid off targeting effects of cytokine administration to patients and may allow for NK cell antitumor functions to be enhanced. Patients with esophageal squamous cell carcinoma, squamous cell lung cancer, and gastric carcinoma have shown positive responses to NK treatments. Higher survival rates were correlated to CD57 positive cells at the site of the tumor. CD57 expression is associated with NK cells, as well as T-cells, and may serve as an additional target for enhancing NK cell effectiveness. Clinical trials to assess the efficacy of NK therapy for gliomas have now been initiated [Table 2] with peer-reviewed reports yet to be released.

**Table 2. Progress using natural killer cells against cancer clinical trials**

Identifier	Trial name	Treatment	Phase	Diagnosis
NCT03383978	Intracranial injection of NK- 92/5.28.z (HER2. taNK) cells in patients with recurrent HER2-positive GBM (Quilt 3.C001) (CAR2BRAIN)	NK-92/5.28.z (HER2. taNK) injection	I	GBM
NCT00909558	Safety and effectiveness study of autologous NK and NK T-cells on cancer	Autologous NK/NK T-cell immunotherapy	I	Glioma, squamous cell lung cancer, pancreatic cancer, colon cancer
NCT00823524	Donor NK cells after donor stem cell transplant in treating patients with advanced cancer	Donor NK cell infusion	I/II	Brain and central nervous system tumors
NCT03081780	Open label NK cell infusion (FATE-NK100) with Subq IL-2 in adults with AML	FATE-NK100	I	Refractory acute myelogenous leukemia, relapsed AML

NK: natural killer; GBM: glioblastoma multiforme; HER2: human epidermal growth factor receptor 2; AML: acute myelogenous leukemia

## CHECKPOINT INHIBITORS

Checkpoint inhibitors are a rapidly advancing field and involve the exploitation of tumor checkpoint regulators. Immune checkpoints regulate the life cycle of the cellular immune response by either activation of signals or by inhibition of activating processes. Tumor checkpoint regulators are mechanisms by which tumors evade immune system recognition through expression of neoantigens. These antigens emulate those of healthy tissue<sup>[39]</sup>. Checkpoint inhibition blocks tumor cell evasion and allows for T-cells to overcome the immunosuppressive tumor microenvironment. However, clinical trial outcomes and patient responses differ between cancer types. Thus, investigation of external influences on checkpoint mechanisms ought to be further explored.

Inhibitors generated for therapeutic use are found as chemically synthesized monoclonal antibodies or recombinant forms of ligands or receptors. Such checkpoint targets include the programmed death receptor 1 (PD-1) and its ligand (PD-L1) or cytotoxic T-lymphocyte associated protein 4 (CTLA-4) receptor and its ligands CD80 and CD86. These pathways are responsible for restriction of T-cells in peripheral tissues during inflammatory response or for down-regulation of co-stimulatory T-cells, respectively<sup>[40-42]</sup>. Although the PD-1 and CTLA-4 pathways are not the only mechanisms which provide cancer cells protection from T-cell surveillance, PD-1 and CTLA-4 have exhibited profound outcomes in regard to tumor regression, appear to possess an immunodominant role as compared to other immune checkpoints, and their mechanisms are the most understood. It has been shown that PD-L1 is highly expressed on tumor cells and that coordination between PD-1/PD-L1 can inhibit CD8<sup>+</sup> T-cell function<sup>[43]</sup>. Administration of PD-L1 inhibitors results in regression of a number of tumor types<sup>[44-49]</sup>. CTLA-4 blockade has shown efficacy in murine melanoma, prostate cancer, and pancreatic carcinoma studies<sup>[50,51]</sup>. The latter demonstrated particular success when combined with PD-1 inhibition, as survival was prolonged even after tumor rechallenge<sup>[52]</sup>. This finding is applicable to cancer cells that remain concealed within the body following tumor resection.

Despite checkpoint inhibitor success in various cancer types, use of this therapy against brain tumors has yet to be extensively pursued. Preclinical assessments in orthotopic, immunocompetent murine models have identified the most effective checkpoint pathway against GBM. When administered alone, PD-1 inhibition has a 50% long term survival rate in mice. Combined treatment with PD-1 and CTLA-4 inhibition was found to achieve 75% long term survival<sup>[53]</sup>. These results paralleled those found in a melanoma clinical trial that utilized the same combination of inhibitors, indicating improved effectiveness<sup>[54]</sup>. Furthermore, checkpoint inhibitor OX-2 glycoprotein (CD200) has been found to be highly expressed in a number of human brain tissue samples, including astrocytomas, meningiomas, and GBM tumors<sup>[55]</sup>. This pathway has been investigated in canine models with high-grade gliomas. Although CD200 canine clinical trials are still ongoing, regression of tumors and absence of inhibitor toxicity has indicated therapeutic promise, and treated groups have already demonstrated an increase of 615 days of survival as compared to control subjects<sup>[56]</sup>. Another pursuit made to target meningioma and other rare CNS tumors is an ongoing, Phase II

**Table 3. Clinical trials using checkpoint inhibitors to treat central nervous system tumors**

Identifier	Trial name	Treatment	Phase	Diagnosis (newly diagnosed or reoccurring)
NCT02866747	A study evaluating the association of hypofractionated stereotactic radiation therapy and durvalumab for patients with recurrent GBM (STERIMGLI)	Durvalumab (PD-L1), radiotherapy	I/II	Reoccurring
NCT02311920	Immune-related DLTs	Pilimumab (CTLA-4), nivolumab (PD-1), TMZ	I	Both
NCT03173950	Immune checkpoint inhibitor nivolumab in people with select rare CNS cancers	Nivolumab (PD-1)	II	Newly diagnosed
NCT02617589	An investigational immuno-therapy study of nivolumab compared to TMZ, each given with radiation therapy, for newly-diagnosed patients with GBM (CheckMate 498)	Nivolumab (PD-1), radiotherapy, TMZ	III	Newly diagnosed
NCT02320058	An investigational immuno- therapy study to evaluate safety and effectiveness in patients with melanoma that has spread to the brain, treated with nivolumab in combination with ipilimumab, followed by nivolumab by itself (CheckMate 204)	Ipilimumab (CTLA-4), nivolumab (PD-1)	II	Both

GBM: glioblastoma multiforme; PD-1: programmed death receptor 1; PD-L1: programmed death receptor ligand 1; DLTs: dose limiting toxicities; CTLA-4: cytotoxic T-lymphocyte associated protein 4; TMZ: temozolomide; CNS: central nervous system

clinical trial utilizing PD-1 inhibitor nivolumab (NCT03173950). This progress emphasizes the importance of continued clinical efforts with checkpoint inhibitors.

Combinatorial methods using checkpoint inhibitors have also been under investigation [Table 3]. Mice implanted with GL261 gliomas treated with both stereotactic radiotherapy and PD-1 inhibitors have shown improved median survival compared to untreated mice. This is thought to be due to increased MHC-I expression and inhibited PD-1 expression, ultimately provoking an increased CD8<sup>+</sup> effector T and decreased Treg population. 15%-40% of mice became long-term survivors, and mice rechallenged with GL261 demonstrated systemic immunity<sup>[57]</sup>. Nivolumab coupled with radiotherapy presents a similar treatment combination, and the therapy is currently being explored in one of the first phase III clinical trials for GBM. The results have not yet been published (NCT02617589). Investigation of anti-PDL1 durvalumab combined with hypofractionated stereotactic radiotherapy to target recurrent GBM has paved the way for a phase II clinical trial, with an absence of serious adverse events and dose-related toxicity related to treatment in patients (NCT02866747). Combined administration of nivolumab and ipilimumab to patients with untreated melanoma metastatic to the brain demonstrated success in a phase II clinical study, with OS rates reaching 92.3% and 82.8% at 6 and 9 months respectively (NCT02320058). Four-1BB is another antibody that prompts CD8<sup>+</sup> and memory T cell proliferation upon activation. A study in mice combining radiation, CTLA-4 blockade, and 4-1BB activation achieved a minimum of 50% long-term tumor free survival, and the treatment increased populations of CD4<sup>+</sup> and CD8<sup>+</sup> tumor infiltrating lymphocytes. Tumor cells were also rejected after re-challenge<sup>[58]</sup>. Finally, a study utilized the catabolic tryptophan enzyme indoleamine 2,3 dioxygenase 1 (IDO), because it is upregulated in 90% of GBM cases, absent in healthy tissue and is also known to play a significant immunosuppressive role in the tumor microenvironment. Combined inhibitors for CTLA-4, PD-L1, and IDO (1-methyl-tryptophan) were administered to mice and resulted in 100% survival<sup>[59]</sup>. Because unperturbed CTLA-4, PD-L1, and IDO pathways greatly augment immunosuppression, it is thought that pathway inhibition should reduce Tregs and result in positive survival outcomes.

One challenge in checkpoint inhibition therapy is identifying which patients might derive the greatest benefit. Prognostic biomarkers must still be defined. The current means of predicting treatment outcome for the PD-1/PD-L1 pathway is by immunohistochemistry of cytologic tumor samples. This method is not completely reliable, as samples are susceptible to contamination and the interpretation of ambiguous findings<sup>[60,61]</sup>. CTLA-4 does not have clinically relevant biomarkers.

Further confounding the process, it is possible that the expression of checkpoint ligands or receptors on tumors may not always be reliable in determining treatment outcomes. In melanoma, for instance, PD-L1

presence on tumors is indicative of survival outcomes, because ligand expression is dependent on CD8<sup>+</sup> T-cells and IFN- $\gamma$  secretion. However, CD8<sup>+</sup> dependence may be specific to melanoma patients, as PD-L1 checkpoint inhibition therapy has demonstrated improved survival with non-small cell lung cancer even if classified as PD-L1 negative<sup>[62]</sup>. Similarly, expression of PD-L1 has not been definitively correlated with prognosis in GBM, suggesting the ligand is not a reliable biomarker<sup>[63]</sup>. Recent work has aimed to explain the immune-resistance of some tumors. Genes in  $\beta$ -catenin, peroxisome proliferator-activated receptor- $\gamma$ , and fibroblast growth factor receptor 3 pathways were found to be responsible for failed T cell priming and recruitment in the urothelial bladder tumor microenvironment in mice. This ultimately led to poor results in checkpoint inhibitor treatment<sup>[64]</sup>. Similar results were found in another study.  $\beta$ -catenin presence in murine BP-SIY tumors is responsible for preventing migration of effector T cells and a robust immune response succeeding ACT<sup>[65]</sup>. In application to transcriptome signatures, these data may allow for more reliable tumor-specific biomarkers options and could improve effectiveness in the total patient population. Because higher mutational load of the tumor has been associated with more effective immunotherapeutic outcomes using checkpoint inhibitors, assays exploring tumor mutational burden are also currently being pursued<sup>[66]</sup>.

These preclinical studies suggest that the mechanism of checkpoint inhibitors is more complex than once thought. Until recently, our gaps in understanding the mechanisms regarding checkpoint inhibition were mostly due to the absence of *in vivo* models representative of the human immune system. However, headway has been made in the development of new models. For instance, we know an exhausted CD8<sup>+</sup> T-cell population surrounds GBM tumors in humans and that this state is achieved through prolonged exposure to the tumor antigen<sup>[67]</sup>. To emulate these conditions in the laboratory, a murine model was generated by infection with chronic lymphocytic choriomeningitis virus followed by induction of murine glioma. This tumor positively responded to anti PD-1 treatment<sup>[68]</sup>. A model for human hematopoietic and immune systems was generated in nonobese diabetic Cg-PrkdcscidIL2rgtm1Wjl/Sz mice by transplantation of human CD34<sup>+</sup> hematopoietic progenitor and stem cells. Patient-derived tumor xenografts in this model responded positively to PD-1 checkpoint inhibitor pembrolizumab<sup>[69]</sup>. Studies utilizing these models should provide a more clear representation of the mechanisms and effects of checkpoint inhibition on human tumors. Through continued efforts, distinct biomarkers can be established for these therapies, and a push for additional clinical trials pursued.

## VACCINE THERAPIES

### Peptide Vaccines

Peptide vaccines have been widely studied for immunotherapy due to their cost-effectiveness, reproducibility, specificity, and low risk of generating an autoimmune response. Peptide vaccines stimulate the immune system by activating CD8<sup>+</sup> and CD4<sup>+</sup> T-cells via APCs. By developing peptides specific to tumors, peptide vaccines can be used to induce an anti-tumor immune response to combat GBM. A limitation of this therapy, however, is the capability of GBM cells to down-regulate MHC-I expression and increase prostaglandin E2 production, which in turn downregulates MHC-II expression on APCs. Furthermore, patient MHC heterogeneity and changes in MHC expression restrict the use of peptide vaccines. To overcome MHC-dependence, long synthetic peptides encoding multiple MHC class I and II epitopes have been developed which are more efficiently processed by DCs and associated with increased CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte activation<sup>[70,71]</sup>.

Antigens used in peptide vaccines can be tumor-specific antigens which are often the products of mutations or splice variants, or tumor-associated antigens which are overexpressed gene products that can be expressed in tumor cells. While tumor-specific antigens result in precise targeting of the tumor, they are not expressed by a majority of the patient population. Conversely, tumor-associated antigens are shared by a larger patient population and have been more preferentially used in vaccines as immunotherapies<sup>[72]</sup>. Adjuvants often supplement antigens to improve immunogenicity. Common adjuvants include PAMPs, damage-associated molecular patterns, or cytokines that can activate APCs and lymphocytes.



**Table 4. Clinical trials using peptide vaccines to treat glioblastoma multiforme**

Identifier	Trial name	Treatment	Status	Diagnosis (newly diagnosed or reoccurring)
NCT02149225	GAPVAC Phase I trial in newly diagnosed GBM patients	Patient-tailored APVAC vaccine plus poly-ICLC and GM-CSF	I	Newly diagnosed
NCT01250470	Phase I study of safety, tolerability and immunological effects of SVN53-67/M57-KOH in patients with survivin-positive malignant gliomas	Montanide ISA-51/survivin peptide vaccine with GM-CSF	I	Both
NCT01222221	A cancer research UK Phase I trial of IMA950 (a novel multi-peptide vaccine) plus GM-CSF in patients with newly diagnosed GBM	IMA-950 vaccine with GM-CSF	I	Newly diagnosed
NCT00626015	Zenapax®-activated peptide immunotherapy	PEP-3 KLH conjugate vaccine with daclizumab	I	Newly diagnosed
NCT00069940	A Phase I study of vaccination with telomerase peptide plus GM-CSF	540-548 telomerase peptide vaccine with GM-CSF	I	Both
NCT00643097	A complementary trial of an immunotherapy vaccine against tumor-specific EGFRvIII	PEP-3 KLH conjugate vaccine with GM-CSF	II	Newly diagnosed
NCT01498328	A Phase II study of rindopepimut/GM-CSF in patients with relapsed EGFRvIII-positive GBM	PEP-3 KLH conjugate vaccine with GM-CSF and bevacizumab	II	Reoccurring
NCT00458601	A Phase II study of CDX-110 with radiation and temozolomide in patients with newly diagnosed GBM	PEP-3 KLH conjugate vaccine with GM-CSF	II	Newly diagnosed
NCT01920191	Phase I/II study of intradermal IMA-950 peptide-based vaccine adjuvanted with intra muscular poly-ICLC in combination with temozolomide in newly diagnosed HLA-A2 GBM patients	IMA-950 vaccine with poly-ICLC	II	Newly diagnosed
NCT01480479	An international, randomized, double- blind, controlled study of rindopepimut/GM-CSF with adjuvant temozolomide in patients with newly diagnosed, surgically resected, EGFRvIII-positive GBM	PEP-3 KLH conjugated vaccine with GM-CSF	III	Newly diagnosed
NCT03422094	A pilot study to assess the safety, feasibility, and immunogenicity of a neoantigen-based personalized vaccine combined with immune checkpoint blockade therapy in patients with newly diagnosed, unmethylated GBM	Neovax with poly-ICLC, nivolumab, and ipilimumab	Active, recruiting	Newly diagnosed
NCT03223103	Phase I study of tumor treatment fields and a personalized mutation-derived tumor vaccine in patients with newly diagnosed GBM	MTA-based vaccine with poly-ICLC and TTF	Active, not recruiting	Newly diagnosed
NCT02455557	A Phase II study of the safety and efficacy of SVN53-67/M57-KLH (SurVaxM) in survivin-positive newly diagnosed GBM	SVN53-67-KLH peptide vaccine with GM-CSF	Active, not recruiting	Newly diagnosed

GBM: glioblastoma multiforme; GM-CSF: granulocyte-macrophage colony-stimulating factor; KLH: keyhole limpet hemocyanin; EGFR: epidermal growth factor receptor; TTF: tumor treating fields; poly-ICLC: polyriboinosinic-polyribocytidylic acid-polylysine carboxymethylcellulose

One of the most successful tumor-specific antigen peptide vaccines against GBM, rindopepimut, uses the EGFRvIII peptide which is expressed in 25%-64% of GBM patients<sup>[17]</sup>. Rindopepimut is commonly co-administered with the keyhole limpet hemocyanin (KLH) and granulocyte-macrophage colony-stimulating factor (GM-CSF) adjuvants. Adjuvants are commonly used to enhance cross-presentation of antigens to improve immunogenicity. A promising phase II trial (ACTIVATE) conducted with rindopepimut and TMZ treatment demonstrated a median OS of 26.0 months [Table 4]. This compared well to controls with a median OS of only 15.0 months (TMZ treatment). Antibody formation against the EGFRvIII peptide was observed in a small subset of patients which correlated with improved median OS, 47.7 months as compared to 22.8 months. However, EGFRvIII negative tumor recurrence was observed in 82% of patients<sup>[73]</sup>. A larger, phase III clinical trial (ACT IV) was later performed that did not show significant improvement in OS following rindopepimut treatment<sup>[74]</sup>. KLH, an adjuvant given with the peptide vaccine, was administered to the phase III control group but not the phase II control group. This suggests that the improved OS may be attributable to the immunogenicity generated by KLH alone and not the peptide. Additionally, at larger scales, peptide vaccinations may be limited by heterogeneity of the patient population. Co-administration of rindopepimut with bevacizumab, however, improved OS for patients which suggests combination with anti-angiogenic therapies may improve efficacy of immunotherapies<sup>[75]</sup>.

Clinical trials involving tumor-associated antigens have not shown significant benefit. Survivin, an inhibitor of apoptosis protein family, is highly expressed in all four subtypes of GBM. A phase I clinical trial found that SurVaxM, a survivin peptide vaccine, did not improve OS, though it was shown to induce cellular and humoral immune responses<sup>[76]</sup> and has moved on to a phase II trial. Similarly, a phase I/II clinical trial using IMA-950, a multi-peptide vaccine, did not significantly improve OS in combination with polyribonucleoside-polyribocytidylic acid-polylysine carboxymethylcellulose and TMZ treatment<sup>[77]</sup>. Similar to SurVaxM, IMA-950 induced a measurable increase in CD8<sup>+</sup> and CD4<sup>+</sup> T-cell response. Lack of correlation between improved outcomes and a peripheral immune response is a common theme among peptide vaccines, and suggests a disrupted interaction between peripheral immune cells and GBM cells due to the immunosuppressive tumor microenvironment.

Recently, efforts have turned to developing personalized peptide vaccines based on analysis of patients' resected tumors. A phase I clinical trial assessed actively personalized vaccination (APVAC) for improving immunogenicity and survival in GBM patients. APVAC induced a CD4<sup>+</sup> T-cell driven immune response in 90% of patients, with a median OS of 29 months<sup>[78]</sup>. However, APVAC was less-tolerated than previous peptide vaccines, with adverse events including anaphylactic reactions and cerebral edema which must be addressed for personalized vaccines to advance.

Overall, peptide vaccines have been shown to induce an immune response without a corresponding improvement in OS. The lack of correlation may be attributed to the immunosuppressive tumor microenvironment. Combination therapies with checkpoint inhibitors may provide a more robust response with better survival outcomes. Personalized vaccine therapies offer a unique and potentially effective way to not only prevent initial tumor progression but also recurrent tumor development, and warrant further investigation. An active clinical trial is currently pursuing combining personalized peptide vaccines with checkpoint inhibitors, and will hopefully elucidate the benefit of these combined therapies (NCT03422094).

### Induced pluripotent stem cells vaccines

Stem cell vaccines comprised of embryonic stem cells (ESCs) have been studied for their ability to generate antitumor immunity. This is largely attributed to the common markers expressed by both tumors and ESCs<sup>[79]</sup>. Studies investigating administration of ESC vaccines prior to tumor induction demonstrated that pre-vaccination could effectively halt tumor growth. However, ethical concerns regarding ESCs limit progress. Focus has now shifted towards induced pluripotent stem cells (iPSCs), which are stem cells derived from somatic cells in combination with Oct3/4, Sox2, c-Myc, and Klf4 transcription factors. The exposure of somatic cells to these transcription factors promotes oncogenic transformation and tumor antigen expression<sup>[80]</sup>. This can lead to improved immunogenicity and more precise targeting of tumor cells. Additionally, iPSCs can be generated from a patient's own tissue and may provide a better representation of a patient's tumor immunogens, although this procedure is not cost-effective.

The use of non-autologous iPSC vaccines can provide a more commercially viable option for iPSC based vaccines. Vaccines generated from iPSCs genetically engineered to express GM-CSF have been shown to suppress lung tumor growth in mice prior to tumor induction as well as in pre-established tumors<sup>[81]</sup>. More recently, an iPSC vaccine comprised of iPSCs with DNA adjuvant CpG demonstrated tumor regression and significantly longer survival in murine models of breast cancer and melanoma. Additionally, treated mice developed antibody titers against iPSCs and tumor cells and were able to protect against tumor rechallenge<sup>[82]</sup>. These data suggest iPSC vaccines may be applicable to other solid tumors, such as GBM.

### DC vaccines

DCs act as the bridge between the innate and adaptive immune system, collecting antigens and presenting them to lymphocytes. DC presentation of antigens to lymphocytes leads to activation of various T-cell populations. These T-cells' respective types and specificity are dependent on the antigens presented by

the DCs and the context in which the DC presents the antigen. To elicit the appropriate lymphocyte and immune response, DCs must present tumor-specific or tumor-associated antigens, upregulate expression of MHC-I and II in conjunction with adhesion and co-stimulatory molecules, and induce secretion of stimulatory and anti-tumor cytokines including IL-12, IL-15, IL-18, and IFN- $\gamma$ <sup>[83]</sup>.

Certain lymphocytes possess inherent capabilities to fight cancer. However, cancers may lack a sufficient supply of tumor antigens to stimulate this immune response. DC vaccines address the lack of tumor antigen by supplying antigen and stimulation to DCs *ex vivo*. Patients can then be vaccinated with these tumor-specific DCs. By manipulating DCs, both in terms of the antigens they present and the context by which they do so, activation of lymphocytes can be manipulated in order to yield an anti-tumor response.

Clinical trials using DC vaccines have employed a variety of antigen loading strategies which include pulsing maturing DCs with autologous-tumor lysate<sup>[84]</sup>. In this technique, DCs must be enriched and matured from monocytes obtained from individual patient's peripheral blood mononuclear cells<sup>[84-87]</sup>. The monocytes are then expanded and differentiated into immature DCs through exposure to GM-CSF and IL-4<sup>[88]</sup>. Immature DCs are loaded with antigens and matured before being administered to patients as a vaccine.

Pulsing maturing DCs with autologous-tumor lysate has made the most progress in GBM therapeutic outcomes. Northwest Biotherapeutics' DCVax<sup>®</sup>-L has recently shown positive results in a phase III clinical trial [Table 5]. Patients selected were between 18 and 70 years of age, and had just been newly diagnosed with GBM. Following surgical resection (the source of autologous tumor-lysate) and chemoradiotherapy, patients were given a series of DCVax<sup>®</sup>-L injections in addition to monthly administration of TMZ. Median OS was 23.1 months, with 25.4% of patients surviving for more than three years post-surgery. This therapy was also well tolerated, with only 2.1% of patients demonstrating grade 3 or 4 adverse events which may have been related to surgery and chemoradiotherapy<sup>[89]</sup>.

Smaller trials using autologous-tumor lysate pulsed vaccines have demonstrated similar safety and efficacy. In the University of Navarra phase II trial, autologous DC vaccines were administered to 31 patients who had a median OS of 23.4 months<sup>[90]</sup>. The high grade glioma-2006 phase I and II trials administered autologous DC vaccines to 77 patients in total with a median OS of 19.4 months<sup>[91]</sup>, and the phase II DENDRI trial administered autologous tumor lysate vaccines to 24 patients demonstrated a median OS of 20.1 months<sup>[92]</sup>. Compared to the 14 months OS following GBM diagnosis and standard of care, DC vaccines pulsed with autologous tumor lysate have demonstrated a substantial and consistent increase in OS, with little to no adverse events.

More recent endeavors have sought to tailor protein-specific DC vaccines through lysates composed of select tumor-associated antigens<sup>[87]</sup> or by transfecting DCs with nucleic acids for tumor-specific or tumor-associated antigen<sup>[93]</sup>, or with cytomegalovirus RNA<sup>[94]</sup>. A phase I study pulsed DC with lysate containing tumor-associated antigens including human epidermal growth factor receptor 2 (HER2), tyrosinase related protein-2, gp100, melanoma-associated antigen 1 (MAGE-1), IL13R $\alpha$ 2, and absent in melanoma 2 (AIM-2), proteins which are enriched in GBM cancer stem cells (GCSC). The multi-epitope-pulsed DC vaccine was used to vaccinate 16 patients with newly diagnosed GBM. Median OS was 38.4 months and improved OS correlated with expression of AIM-2 and MAGE-1 in the tumor. Notably, a decrease was seen in GCSC marker CD133, suggesting that the multi-epitope-pulsed DC vaccine had successfully reduced the population of cancer stem cells<sup>[87]</sup>, potentially accounting for the significant increase in median OS, nearly three times that seen with the standard of care.

Transfection of DC to induce expression of tumor-specific or tumor-associated proteins has also demonstrated success in treating GBM. In a phase I clinical trial GCSC mRNA was isolated from brain

**Table 5. Clinical trials using dendritic vaccines to treat gliomas**

Identifier	Trial name	Treatment	Phase	Diagnosis (newly diagnosed or reoccurring)
NCT00107185	Vaccine therapy in treating young patients who are undergoing surgery for malignant glioma	Autologous DC vaccine	I	Both
NCT01171469	Vaccination with dendritic cells loaded with brain tumor stem cells for progressive malignant brain tumor	Autologous DC vaccine	I	Both
NCT00639639	Vaccine Therapy in treating patients with newly diagnosed GBM (ATTAC)	DC vaccine with mRNA from human cytomegalo-virus	I	Newly diagnosed
EudraCT 2006- 002881-20	HGG-2006 phase I/II trial	Autologous DC vaccine	I/II	Newly diagnosed
NCT00846456 (EudraCT 2007- 006171-37)	Safe study of DC based therapy targeting tumor stem cells in GBM	DC vaccine with mRNA from tumor stem cells	I/II	Both
NCT00323115	Phase II feasibility study of DC vaccination for newly diagnosed GBM	Autologous DC vaccine	II	Newly diagnosed
EudraCT 2008- 005035-15	DENDR1	Autologous DC vaccine	II	Newly diagnosed
EudraCT 2008- 005038-62	DENDR2	Autologous DC vaccine	II	Reoccurring
NCT03395587	Efficiency of vaccination with lysate-loaded DCs in patients with newly diagnosed GBM (GlioVax)	Autologous DC vaccine	II	Newly diagnosed
NCT01006044 (EudraCT 2009- 009879-35)	Efficacy & safety of autologous DC vaccination in GBM after complete surgical resection	Autologous DC vaccine	II	Newly diagnosed
NCT01280552	A study of ICT-107 immunotherapy in GBM	Multi-epitope pulsed DC vaccine	II	Newly diagnosed
NCT02546102	Phase 3 randomized, double-blind, controlled study of ICT-107 in GBM	Multi-epitope pulsed DC vaccine	III	Newly diagnosed (and in remission)
NCT00045968	Study of a drug [DCVax®-L] to treat newly diagnosed GBM brain cancer	Autologous DC vaccine	III	Newly diagnosed

DC: dendritic cell; GBM: glioblastoma multiforme; HGG: high grade glioma

tumor biopsies and transfected into DC cells used to vaccinate patients. To isolate GCSC, patient tumor cells were selected for their ability to form spheres *in vitro* as a proxy for identifying cancer stem cells. From the sphere-forming tumor cells, mRNA was isolated and transfected into patient derived DCs, which were used as vaccinations for seven patients. Patients showed no adverse effects and had a median OS of 23 months<sup>[95]</sup>, comparable to that seen in DC vaccines loaded with autologous tumor-lysate.

In addition to cellular proteins, recent studies have shown that a high percentage of GBM express cytomegalovirus proteins, a potential target for DC vaccines. In a phase I clinical trial, 11 patients were vaccinated with DCs transfected with cytomegalovirus pp65 lysosome-associated membrane glycoprotein mRNA, following resection and radiochemotherapy. These patients demonstrated a median OS of 41.1 months, with no adverse effects attributed to the cellular component of treatment<sup>[94]</sup>. If larger studies confirm that a significant number of GBM patient tumors exhibit cytomegalovirus, cytomegalovirus proteins offer a clear target for DC vaccines, and potentially peptide vaccines as well.

One challenge of evaluating DC vaccines has been the lack of consistency across trials to measure immune response. While median OS provides a good measure of general effectiveness, trials vary in measurements when evaluating immunological outcomes, such as cytokine levels or immune cell counts. Trials have shown mixed results as to whether or not DC vaccines increase cytotoxic T-cell and TH1 responses, which is further confounded by trials that did not measure these criteria. Trials that have measured NK cell populations have shown that an increase in NK cell activity and number following vaccinations has been correlated with improved outcomes<sup>[86,92]</sup>. In order to improve the development of DC vaccinations, consistent immunological evaluations (patient T-cell, NK cell, and cytokine production, *etc.*) offer a clear target so that the mechanisms underlying the success of various aspects of DC vaccinations can be elucidated and better applied going forward.

While DC vaccines have shown their ability to prolong OS, correlation of immune response with long-term outcomes remains unclear, and prognostic markers for determining patients that will best respond to DC vaccines needs to be further elucidated. DC vaccines targeting specific proteins, the multi-epitope trial and cytomegalovirus trial, show great clinical potential, but must be evaluated on a larger scale before conclusions can be drawn. Whole-tumor lysate vaccines are the most advanced in terms of clinical trial progress and demonstrate clear improvements, with 25% of patients achieving above a three-year survival rate in the largest phase 3 trial to date. This trial further demonstrated increased efficacy in patients with methylated O6-methylguanine-DNA methyltransferase, offering another potential prognostic marker for identifying patients who might best respond to autologous tumor-lysate DC vaccines<sup>[89]</sup>. For patients whose tumors can be biopsied, DC vaccines offer a significant improvement in survival outcome, which will be enhanced as prognostic markers become clearer.

## CONCLUSION

Glioma-targeted immunotherapy is still in its infancy. Although ACT, NK cells, checkpoint inhibitors and vaccines have proven their efficacy in other cancers, a deeper understanding of the features specific to solid gliomas is necessary for refined therapy adjustment. Furthermore, improved human preclinical models can more accurately illustrate the human CNS microenvironment and immune cell relationships with the BBB. Studies utilizing these models can deepen our understanding of immune function, ultimately revealing ways to enhance combined treatment modalities. Yet as these ambiguities are made clear, the future of these treatments against GBM remains bright. These methods of tumor eradication address limitations posed by conventional surgical, radiation, and chemotherapies.

## DECLARATIONS

### Authors' contributions

Conceptualize the project, write and edit the manuscript: Low WC, Crane AT, Pearce CM, Chrostek MR  
Write various sections of the manuscript: Fellows EG, Toman NG, Tran S

### Availability of data and materials

Not applicable.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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Case Report

Open Access



# Paraneoplastic limbic encephalitis associated with testicular mixed germ cell tumor

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## Abstract

Paraneoplastic limbic encephalitis (PLE) is a rare immunopathological syndrome, reported in association with certain types of malignancies. Patients present with cognitive and memory impairments, disordered perception, mood and behavioral changes, sleep disturbances, and seizures. Despite the growing number of cases being reported, it still poses a diagnostic challenge. We encountered a patient with a myriad of neuropsychiatric symptoms who exhibited a highly variable response to therapy. A 36-year-old male presented with memory impairment, excessive sleepiness, and slurred speech. Brain magnetic resonance imaging revealed hyperintensities in the temporal lobes and hypothalamus, all suggestive of limbic encephalitis. He was found to have a mixed germ cell testicular teratoma. Screening for commonly associated antibodies did not yield positive results, which emphasizes that sero-negative PLE can be missed in patients with malignancies. In reporting this case, we urge neurologists to consider PLE as part of the differential diagnosis in similar ambiguous clinical scenarios.

**Keywords:** Paraneoplastic syndrome, limbic encephalitis, testicular mixed germ cell tumor

## INTRODUCTION

Paraneoplastic limbic encephalitis (PLE) is a rare syndrome characterized by confusion of acute onset, mood changes, hallucinations, loss of short term memory, and seizures<sup>[1]</sup>. The pathogenesis is not fully understood but is thought to be due to autoimmune cross reactivity.



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This is a case involving a patient who presented with an acute state of confusion and sleeping attacks. There was an incidental finding of a left testicular tumor and brain magnetic resonance imaging (MRI) showed signs of limbic encephalitis. The patient was later diagnosed with PLE secondary to testicular mixed germ cell tumor; we are reporting a rare case of PLE due to testicular mixed germ cell tumor.

The aim is to guide neurologists to recognize the clinical manifestations of neurologic paraneoplastic disorders, more specifically the PLE subtype, and to distinguish them from other causes of neurologic dysfunction in cancer patients. Early diagnosis of paraneoplastic syndromes can maximize the likelihood of favorable oncologic and neurologic outcome<sup>[2]</sup>.

## CASE REPORT

A 36-year-old right-handed Saudi male, with a medical history of hypertension and liver transplantation in 1995 maintained on tacrolimus, presented to the emergency department with a complaint of progressive impairment in recent memory, excessive sleepiness, slurred speech, and depression for 3 weeks with an increase in severity over the past few days. Neurologic examination revealed unbalanced gait but otherwise motor and sensory functions were within normal limits. There was no history of abnormal movement, tongue biting, headache, neck pain or nuchal rigidity, fever, visual disturbances, weakness, sensory deficits, sphincter dysfunction, or head trauma. In addition, there was no history of smoking, alcohol, or drug abuse.

On examination, the patient was vitally stable with a blood pressure 153/68 mmHg and oxygen saturation 100% on room air, and afebrile. He was conscious, alert, inattentive, and disoriented to time and place. His Glasgow Coma Scale was 14/15. His pupils were 2 mm in size, with a sluggish reaction to light and vertical gaze palsy. Mini mental state examination showed moderate cognitive impairment of 10.

Extensive biochemical, hematological, and radiological investigations were carried out. The basic laboratory tests were not suggestive of any extracranial or infectious causes. Tumor markers screened included alpha-fetoprotein 229 ng/mL which was markedly elevated. However, beta-human chronic gonadotropin and carcinoembryonic antigen were of normal levels.

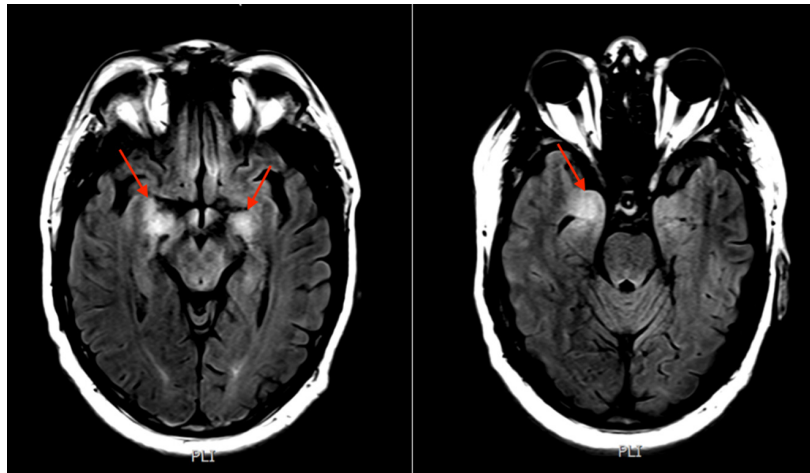
On admission, the patient was started on IV methylprednisolone (1 g/day for 5 days), and acyclovir (1.2 g for 1 day) until herpes encephalitis was ruled out. His level of consciousness improved with the administration of steroids. However, he became agitated and developed visual hallucinations. These symptoms were managed using haloperidol 2.5 mg intravenous and risperidone 0.5 mg orally.

Brain computed tomography (CT) without contrast was normal. Brain fluid-attenuated inversion recovery (FLAIR) MRI [Figure 1] revealed abnormal signals involving the medial temporal lobes and hypothalamus suggestive of limbic encephalitis. Cerebrospinal fluid was colorless and its analysis showed mild lymphocytosis with a glucose level of 8.1 mmol/L, protein of 0.57 g/L, white blood cell count of 30 cells/ $\mu$ L mostly lymphocytes. Central nervous system infection and metastasis were excluded.

With this clinical picture, paraneoplastic syndrome was in the top differentials and we sought to rule out common cancers via investigating for tumor markers and imaging. CTs of the chest and abdomen, in addition to renal, abdominal, and testicular ultrasounds were done. Testicular ultrasounds [Figure 2] revealed a well-defined heterogeneous focal mass with cystic changes and micro-calcification in the left testicle. The mass measured 5 cm by 2.7 cm in size. Pan-CT was performed pre- and post-contrast using a triphasic study, and the scan showed multiple retroperitoneal lymph nodes that are most likely related to the testicular tumor, with no suggestion of thoracic or abdominal metastasis.

The patient was now diagnosed with PLE secondary to testicular cancer. Subsequently, screening for common antibodies associated with paraneoplastic encephalitis including anti-Ma2, anti-N-Methyl-D-





**Figure 1.** Fluid-attenuated inversion recovery magnetic resonance imaging of the brain showing hyperintensity within the medial aspect of the temporal lobes suggestive of limbic encephalitis

aspartic acid (NMDA), anti-voltage-gated potassium channels (VGKC), anti-contactin associated protein 2 (CASPR2) and anti-LG1 was conducted, but all yielded negative results.

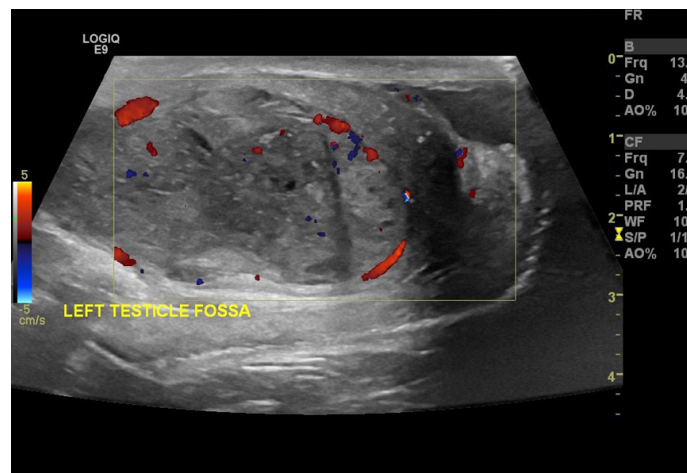
Due to his neurological state at the time, our patient was deemed an unfit candidate for chemotherapy, and tumor resection was the mainstay approach for treating the cancer. Left radical inguinal orchiectomy was done and revealed the mass to be a mixed germ cell tumor constituting yolk sac tumor 90% [Figure 3] and teratoma 10% [Figure 4]. The resection was intended to treat the cancer itself and halt the source of the neurological symptoms.

The oncology team decided against adjuvant chemotherapy after the resection, and were reassured by the regression in lymph node sizes. His level of consciousness improved and his speech became more clear. However, there was no improvement in his memory, orientation, or gaze. In the following days, he returned to his baseline status upon presentation where he experienced excessive sleepiness and more visual hallucinations. The patient underwent multiple sessions of pulse steroid therapy, intravenous immunoglobulin (IVIG) and rituximab but there was no clinical improvement. Moreover, the patient started to develop seizures after the initial IVIG session and was then switched to rituximab. The seizures were slow with no epileptiform discharges, and despite the change in treatment they recurred frequently.

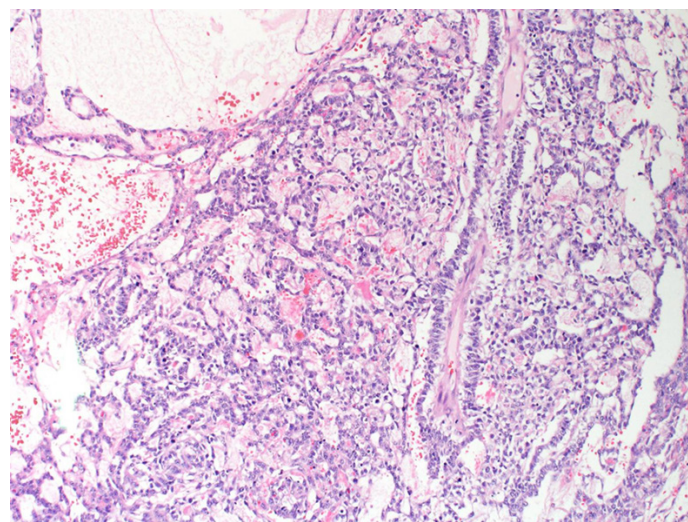
In the subsequent months, our patient continued to deteriorate in terms of level of consciousness and respiratory function. He was admitted to the intensive care unit (ICU) where he later became dependent on mechanical ventilation. During his stay in the ICU, he developed multiple septic shocks due to his impaired immunity from tacrolimus as an attempt to prevent liver transplant rejection and unfortunately later went into multi-organ failure. He passed away from cardiac arrest.

## DISCUSSION

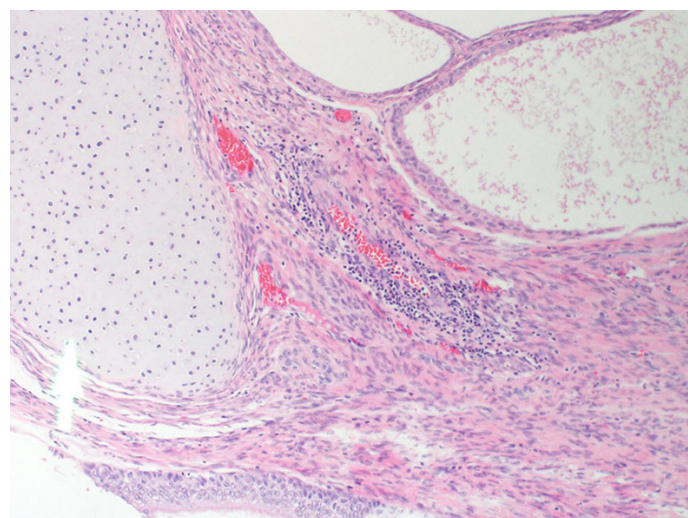
Limbic encephalitis has been first described as a paraneoplastic syndrome by Corsellis *et al.*<sup>[3]</sup> in 1968. Considered to be a rare association of common malignancies, such as small cell lung cancer, testicular and ovarian cancers<sup>[4]</sup>, Hodgkin's lymphoma, thymoma, and teratomas<sup>[5]</sup>. The incidence depends on the type of associated cancer; with the lowest incidence of 8% attributed to testicular cancer, as described by Gultekin *et al.*<sup>[1]</sup>. This incidence is expected to increase with the development of new diagnostic methods and treatment modalities, which will lead to prolonged survival of cancer patients and hence increase the number of diagnosis of such cases.



**Figure 2.** Ultrasounds picture showing left testicular mass with cystic changes and microcalcifications, neoplastic



**Figure 3.** Yolk sac component of the tumor displaying endodermal sinus, reticular, microcystic, and solid patterns with prominent Schiller Duval bodies



**Figure 4.** Teratomatous epithelium (cartilage and pulmonary tissue), along with mature nervous tissue

Neurologic paraneoplastic disorders are believed to result from an immune cross-reactivity between the tumor cells and components of the nervous system<sup>[6]</sup>. PLE has been associated with auto-antibodies produced against neuronal cell surfaces and synaptic bodies, expressed in nervous tissue and tumors<sup>[7,8]</sup>. Their presence indicates an underlying tumor and often allows early detection of the associated tumor, thus aiding the diagnosis. However, these antibodies are detected in only 60% of patients diagnosed with PLE<sup>[9]</sup>. Commonly encountered antibodies include anti-NMDA, anti-Ma2, anti-Hu, anti-Ta, anti-LG1, anti-CASPR2 and anti-VGKC<sup>[8]</sup>.

PLE is a spectrum of heterogeneous neuropsychiatric manifestations, described as a result of the inflammation of the limbic system. The constellation of symptoms include acute or subacute mental confusion, impairment of recent memory, cognitive dysfunction, hallucinations, depression, personality changes, complex partial seizures and sleep disturbances<sup>[10]</sup>. Hyperthermia, somnolence, and endocrine abnormalities may also occur as a result of hypothalamic involvement<sup>[4]</sup>. Although the inflammation is localized to the limbic system, Newman *et al.*<sup>[8]</sup> explains the clinical and pathologic findings are not confined to these anatomical areas, and symptoms of brainstem and cerebellar dysfunctions commonly occur, as exhibited by the unbalanced gait of our patient.

The non-specific nature of neuropsychiatric manifestations often precedes the detection of cancer in 80% of patients<sup>[11]</sup>, presents a diagnostic challenge. In addition, its mimicry of other presentations such as viral encephalomyelitis or rapidly progressive neurodegenerative disease, broaden the differential diagnosis<sup>[2]</sup>. Therefore, tumor screening via imaging and electroencephalography (EEG) is paramount when unexplained paraneoplastic neurologic symptoms are encountered. This uncovered the masked diagnosis of testicular mixed germ cell tumor in our patient, despite the unrevealing results of auto-antibodies.

Although imaging results are often nonspecific, MRI with T2/FLAIR remains the most sensitive tool to diagnose PLE. Medial temporal lobe hyperintensities are seen with infrequent contrast enhancement<sup>[4]</sup>. EEG supports the diagnosis by showing focal or generalized slowing and/or epileptiform activity, also maximal in the temporal regions. It is useful for determining alterations in consciousness due to postictal states or partial complex seizures<sup>[5]</sup>.

Diagnostic dependence on antibody testing and immunotherapy response might delay proper detection according to Graus *et al.*<sup>[7]</sup>, since several antibody tests may not be widely available in facilities. These tests require a long duration to produce results, and interestingly, the absence of the antibodies does not exclude the diagnosis and vice versa<sup>[6,7]</sup>.

Lancaster<sup>[2]</sup> argues the use of response to immunotherapy as an indicator of the diagnosis can also be misleading as some patients may require intensive treatments not available in all centers or may not respond at all. Moreover, patients with other diagnoses may show clinical improvement. The growing consensus advises to start empiric immunotherapy before antibody testing in suspected autoimmune encephalitis<sup>[7]</sup>.

Given the novelty and variation in responses, definitive treatment is not yet available. Early diagnosis aids in the eradication of the underlying cause and antigen source, and in turn provides the highest chance of neurological improvement, with the best prognosis. Suppression of this overactive immune response is also a cornerstone in treatment; achieved by an array of medical modalities, including steroids, cyclophosphamide, IVIG, and plasma exchange<sup>[8]</sup>.

Despite the successful eradication of the tumor in our patient, neurologic improvement was not guaranteed because the causative agents were not produced by the tumor cells, and the possible permanent damage accrued by the immune response. This emphasizes that all efforts should be extended in finding the hidden

neoplasm to initiate early treatment and potentially controlling this disease.

## DECLARATIONS

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### Authors' contributions

Concept, literature search preparation, editing and revision of manuscript: Alsaffar G, Almedallah D, Al-Shabeeb G

Patient data and imaging acquisition: Baarmah A

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### Availability of data and materials

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IRB approval from the hospital and a written informed consent were obtained.

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Not applicable.

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Review

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# Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy vs. multiple sclerosis. Either one or sometimes both?

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## Abstract

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL), is the most common cause of inherited cerebral small vessel disease, inherited stroke and inherited vascular dementia. It is not infrequent for CADASIL to be mistaken and mistreated for multiple sclerosis (MS). A much less frequent but existing scenario is the co-occurrence of CADASIL and MS (or MS-like inflammatory condition). Such patients may present with spinal cord lesions, brain or spinal cord enhancing lesions, positive oligoclonal bands and high IgG index in the cerebrospinal fluid and good response to corticosteroids or immunomodulating treatments. CADASIL through various mechanisms may trigger or modulate autoimmune reactions, and either be complicated by an inflammatory component or cause an MS-like disorder.

**Keywords:** Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy, multiple sclerosis, autoimmunity, NOTCH3

## INTRODUCTION

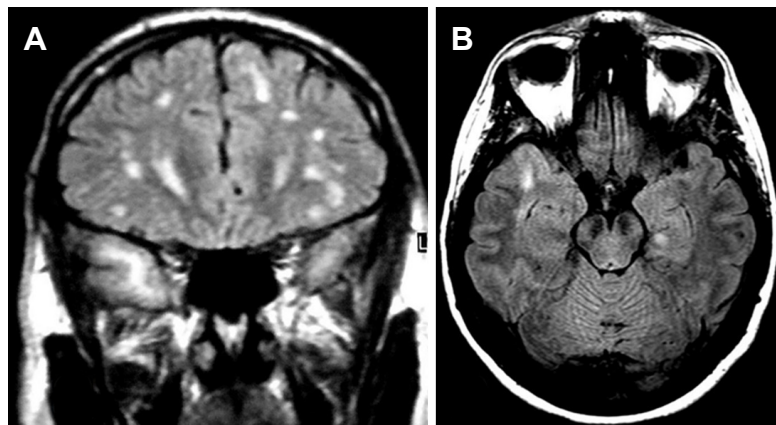
Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL), caused by mutations in *NOTCH3* gene<sup>[1]</sup> is the most common cause of inherited cerebral small vessel disease, inherited stroke and inherited vascular dementia<sup>[2]</sup>. Patients typically present with various combinations of



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**Figure 1.** Magnetic resonance imaging of two patients (Fluid Attenuated Inversion Recovery images). (A) A 38-year-old male with CADASIL and multiple hyperintense lesions. Due to his age, they were initially thought by the radiologist to represent MS. However, anterior temporal lobe lesions prompted genetic testing, revealing the correct diagnosis; (B) The opposite may also occur. This 31-year-old female with the right anterior temporal lesion suffers from relapsing-remitting MS with oligoclonal bands and high IgG index in the CSF. Genetic testing of *NOTCH3* was negative. CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy; MS: multiple sclerosis; CSF: cerebrospinal fluid

migraine with aura, stroke or transient ischemic attacks (TIAs), in the presence of multiple and progressively confluent white matter ischemic lesions involving, among others, the anterior temporal lobe and the external capsule<sup>[3]</sup>. As the load of ischemic lesions increases and the disease progresses, behavioral and psychiatric symptoms, vascular dementia and motor disability become evident<sup>[4]</sup>. Atypical presentations may exist and the clinical differential diagnosis may be extensive<sup>[5]</sup>. Diagnosis is confirmed by genetic testing of the *NOTCH3* gene<sup>[1,4]</sup>.

### CADASIL MISDIAGNOSED AS MULTIPLE SCLEROSIS

It is long known that, during the early or middle stages of CADASIL, recurrent ischemic events and multiple white matter lesions may mimic multiple sclerosis (MS)<sup>[6]</sup> [Figure 1]. Autosomal dominant inheritance, migraine with aura, characteristic involvement of the anterior temporal lobe, ischemic lesions within the basal ganglia and deep nuclei such as the thalamus and, later on, dementia may point towards CADASIL. On the contrary, lesions present exclusively in the white matter (especially the periventricular white matter) and the corpus callosum, spinal cord involvement, history of optic or retro bulbar neuritis and the additional presence of oligoclonal bands in the cerebrospinal fluid, may point towards MS [Table 1]. However, some of the above may be absent, unrecognized, atypical or not reported. Patients with *de novo* *NOTCH3* mutations and, thus, with negative family history have been described<sup>[7]</sup>, while familial forms of MS are known to exist and enter in the differential diagnosis<sup>[5]</sup>. Migraine with aura or the characteristic anterior temporal lesions may be absent in almost 50% and 25% of CADASIL patients respectively<sup>[8]</sup> and these percentages may be even larger in patients of Chinese origin<sup>[9]</sup>. Corpus callosum or pericallosal lesions (typically present in MS) may also occur in CADASIL<sup>[3,10-12]</sup>. Atypical features may increase diagnostic confusion and increased clinical suspicion may be needed to prompt diagnostic testing of *NOTCH3*.

Despite the better understanding of typical and atypical clinical presentations of CADASIL gained overtime and the availability of genetic testing, misdiagnosis of CADASIL as MS is still not an infrequent scenario<sup>[13,14]</sup>. Even in typical cases, prolonged mistreatment of CADASIL with immunological treatments targeting MS has been described<sup>[14]</sup>. An older study revealed no association between *NOTCH3* mutations and MS<sup>[15]</sup>. This led some authors to adapt the notion that the differential diagnostic question has a dichotomous answer, i.e. the patient has either CADASIL or MS<sup>[14]</sup>. This may not be always true. A rare but existing scenario implicates the presence of autoimmune (including MS-like) disorders, in patients with CADASIL.

**Table 1. Features useful in the differential diagnosis between CADASIL and MS**

	CADASIL	MS
<b>Clinical features</b>		
Autosomal dominant inheritance	Usually present	Usually absent
Migraine with aura	Increased frequency compared to the general population	Same frequency with the general population
Recurrent neurological symptoms	Ischemic	Demyelinative
Vascular dementia	Yes	Usually absent (cognitive and psychiatric symptoms may exist)
<b>Neuroimaging findings</b>		
Characteristics of white matter lesions	Initially focal, progressively confluent, tend to spare the U fibers	Oval lesions perpendicular to the lateral ventricles Gadolinium enhancing lesions
Involvement of the temporal pole	Usually present	Usually absent
Involvement of the external capsule	Usually present	Usually absent
Involvement of corpus callosum	May be present	Usually present
Involvement of deep subcortical nuclei (basal ganglia, thalamus)	Yes	No
Spinal cord involvement	Extremely rare	Frequent
Hemorrhagic lesions (usually microbleeds)	May be present	Absent
<b>CSF immunology</b>		
Oligoclonal bands (unmatched in serum)	Absent	Present
IgG index	Normal	Increased

CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy; MS: multiple sclerosis; CSF: cerebrospinal fluid

## PATIENTS WITH CADASIL AND VARIOUS AUTOIMMUNE DISORDERS

The possible involvement of autoimmune mechanisms in some patients with CADASIL has been hypothesized<sup>[16]</sup>. The presence of antiphospholipid antibodies has been reported in two unrelated female patients with CADASIL, suggesting that the two conditions may co-occur<sup>[17]</sup>. Central nervous system angiitis may also co-occur with CADASIL<sup>[18]</sup>. The presence of antinuclear antibodies has been reported in at least 3 members of a CADASIL family, one of which was also positive for anti-SSA and anti-SSB antibodies<sup>[11]</sup>. Autoimmune thrombocytopenia has been observed in an elderly patient, leading to aspirin discontinuation and stroke recurrence<sup>[19]</sup>. Renal involvement with IgA mesangial deposition in addition to the typical granular osmiophilic material of CADASIL has been reported in patients from unrelated families with *NOTCH3* mutations, leading to the diagnosis of CADASIL complicated with IgA nephropathy<sup>[20,21]</sup>.

The above observations indicate that autoimmune conditions may rarely coexist with CADASIL and the presence of one should not preclude the diagnosis of the other.

## POSSIBLE COMORBIDITY OF MS AND CADASIL

Some patients with genetically proven CADASIL may present with MS or MS-like conditions. Oligoclonal bands in the cerebrospinal fluid (CSF), a characteristic finding in MS, are extremely uncommon in CADASIL, but they have been reported<sup>[22,23]</sup>. The occurrence of spinal cord lesions, especially longitudinal ones, are exceedingly rare in CADASIL and may be due to ischemia<sup>[24]</sup> but, when present, they evoke a diagnostic challenge<sup>[25]</sup>. In one family with CADASIL, 3 members presented with cord lesions in the posterocentral area, cerebral lesions in locations compatible with both demyelination and typical CADASIL, positive antinuclear antibodies and CSF oligoclonal bands<sup>[26]</sup>. Another CADASIL patient with thoracic cord involvement, in the absence of CSF oligoclonal bands, showed paramagnetic enhancement of an internal capsule lesion and good response of his gait disorder to corticosteroids<sup>[27]</sup>. A ring enhancing lesion in the cerebellar peduncle and a solid enhancing lesion in the corona radiata were observed in a patient positive for CSF oligoclonal bands and with a high IgG index, who later developed new multiple enhancing lesions and new cervical spinal lesions<sup>[28]</sup>.

Relapsing optic neuritis without CSF oligoclonal bands has been described in a CADASIL patient with sensorimotor leg deficit, showing good response to corticosteroids and later to glatiramer acetate<sup>[27]</sup>. According to the authors, this patient would otherwise fulfill the diagnostic criteria of MS.

Balo concentric sclerosis has been reported in a patient carrying a *NOTCH3* mutation<sup>[29]</sup>. Oligoclonal bands were absent and response to corticosteroids was minimal. However, significant improvement was noted after 5 sessions of plasmapheresis. He was then treated as a clinically isolated syndrome with  $\beta$ -interferon-1a and no relapse was noted for at least 1 year.

The above reports of cases or families indicate that, rarely, MS or MS-like conditions may coexist with CADASIL. The presence of CSF oligoclonal bands or high IgG index and/or the responsiveness to immunological treatments points towards the autoimmune nature of these conditions. They may represent true MS or some other related demyelinating disease or an inflammatory form/component of CADASIL<sup>[27-29]</sup>. The co-occurrence of the two conditions could be coincidental. But, is there a possibility that *NOTCH3* mutations may somehow provoke (or at least alter) autoimmune phenomena?

## THE INTERPLAY BETWEEN CADASIL AND AUTOIMMUNITY

It has been hypothesized that an interaction may be normally present between the immune and *NOTCH3* signaling systems. This interaction may be altered by *NOTCH3* mutations or Notch3 dysfunction<sup>[30,31]</sup>. In addition, the gain of a novel/toxic function of the mutant Notch3 protein, suggested to occur in CADASIL<sup>[1,4]</sup>, may be associated, through altered protein-protein interactions, with immune dysregulation and/or presentation of new epitopes<sup>[27]</sup> leading to autoimmunity. This presentation of new epitopes may be facilitated by abnormal aggregations of extracellular matrix proteins, forming complexes with the extracellular domain of the Notch3 receptor<sup>[32]</sup>. Dysregulation of T-cells by altered Notch3 function may offer an additional or alternative mechanism for inflammatory demyelination<sup>[31]</sup>.

Alterations of the vascular wall occurring in CADASIL<sup>[4]</sup> are thought to affect the blood brain barrier and, indeed, many patients have CSF evidence of blood brain barrier dysfunction (increased protein)<sup>[23]</sup>. Such a dysfunction, possibly augmented by ischemia, may expose central nervous system antigens to the immune system, initiating an autoimmune reaction<sup>[28]</sup>. It seems possible however, that the *NOTCH3* mutations alone are not enough to trigger autoimmunity since, most patients do not have such coexisting disorders. Other genetic or epigenetic factors may participate.

CADASIL is not the only genetic encephalopathy, which may (rarely) present with autoimmune comorbidity or be confused with MS. Indeed, some genetic white matter diseases, including adrenoleucodystrophy, may be complicated by an inflammatory component during disease progression<sup>[5]</sup>. Interestingly enough, some other genetic conditions may have both an ischemic and an inflammatory component, they may be accompanied by migraine with aura and ischemic events (including stroke or stroke-like episodes) and they may result in cognitive impairment and psychiatric/behavioral symptoms. Retinal vasculopathy with cerebral leucodystrophy (RVCL), due to mutations of *TREX1* is one such disorder, presenting with many, sometimes overlapping phenotypes<sup>[33]</sup>. Renal and retinal involvement, together with contrast-enhancing mass lesions in brain imaging, distinguish this disorder from CADASIL. Mitochondrial diseases including MELAS<sup>[34]</sup>, *POLG*-related<sup>[35]</sup>, and *OPA1*-related<sup>[36]</sup> disorders may sometimes have an autoimmune component.

## CONCLUSION

It is not infrequent for CADASIL to be mistaken for MS<sup>[37]</sup>. Better understanding of the disease is required for clinical neurologists and radiologists, in order to avoid diagnostic pitfalls and mistreatments. A much less frequent, yet existing scenario, is the co-occurrence of CADASIL and MS (or MS-like inflammatory

condition). This co-occurrence may be incidental and the two disorders may progress in parallel, but unrelated to each other. Alternatively, the two disorders, once incidentally coexistent, may interact with each other, modulating the pathophysiological mechanisms and phenotypes. A third possibility is that CADASIL through various mechanisms may trigger autoimmune reactions, and either be complicated by an inflammatory component (“inflammatory form of CADASIL”) or cause an MS-like disorder.

Mistreating CADASIL with immunomodulating treatments targeting MS should be avoided. However, the rare CADASIL patient with an inflammatory component should not be denied the use of immunological treatments.

## DECLARATIONS

### Authors' contributions

Concept and definition of intellectual content: Paraskevas GP, Kapaki E

Literature search: Paraskevas GP, Constantinides VC

Manuscript preparation: Paraskevas GP, Constantinides VC

Manuscript editing and manuscript review: Kapaki E

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declare no conflicts of interest.

### Ethics approval and patient consent

This review is part of the study named “Migraine and Specific Vasculopathies Registry” of the 1st Department of Neurology, National and Kapodistrian University of Athens, which has been approved by the Scientific and Ethics Committee of Eginition Hospital and is not supported by any funding. The patients whose MRIs are shown in Figure 1, gave informed consent for inclusion in the study and publication of their MRI images.

### Consent for publication

Not applicable.

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Original Article

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# Interleukin-1 $\beta$ -induced inflammatory signaling in C20 human microglial cells

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## Abstract

**Aim:** Increased inflammatory signaling in microglia is implicated in the pathogenesis of neurodegenerative diseases, trauma, psychiatric disorders, and anxiety/depression. Understanding inflammatory signaling in microglia is critical for advancing treatment options. Studying rodent-derived microglia has yielded substantial information, yet, much remains to better understand inflammatory signaling in human microglia. Hence, there is great interest in developing immortalized human microglial cell lines. The C20 human microglial cell line was recently developed and our primary objective was to advance our knowledge of inflammatory signaling in these cells.

**Methods:** Expression of the microglia specific marker transmembrane protein 119 (TMEM119) was assessed by western blot analysis. Lipopolysaccharide (LPS)- and interleukin-1 $\beta$  (IL-1 $\beta$ )-induced cytokine/chemokine expression was determined by ELISA. Phosphorylation of inhibitory kappa B alpha (I $\kappa$ B $\alpha$ ), nuclear factor (NF)- $\kappa$ B p65, and p38 mitogen-activated protein kinase (p38 MAPK) was measured by western blot analysis.

**Results:** TMEM119 was expressed in unstimulated C20 cells, and to a greater extent in IL-1 $\beta$ -stimulated cells. IL-1 $\beta$  significantly induced IL-6, monocyte chemoattractant protein-1/CCL2, and interferon- $\gamma$  inducible protein 10/CXCL10 expression. LPS induced CCL2 expression, but not IL-6 or CXCL10 expression. IL-1 $\beta$  induced inflammatory signaling as indicated by increased phosphorylation of I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65 and p38 MAPK.

**Conclusion:** We provide the first evidence that C20 microglia express TMEM119. This is the initial report of IL-1 $\beta$ -induced activation of I $\kappa$ B $\alpha$ , NF- $\kappa$ B p65, and p38 MAPK and subsequent CXCL10, CCL2 and IL-6 secretion in C20



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cells. These findings advance our understanding of inflammatory signaling in C20 cells and support the value of this cell line as a research tool.

**Keywords:** Interleukin-1 $\beta$ , chemokine, microglia, p38, nuclear factor- $\kappa$ B p65, neuroinflammation

## INTRODUCTION

Microglia are resident macrophages in the central nervous system (CNS) and are essential to brain physiology; but also, they are instrumental in response to injury and infection in the CNS (See Wolf *et al.*<sup>[1]</sup> for review). Microglia constantly survey their local environment and respond to extracellular cues (e.g., ATP) to maintain homeostasis<sup>[2,3]</sup>. Other specific physiological functions of microglia include removal of dead neurons and cellular debris<sup>[2,3]</sup>, synaptic pruning<sup>[4]</sup>, and regulation of synaptic connectivity and plasticity<sup>[5-7]</sup>. Microglia are also integral to innate immunity and are instrumental in neuroinflammation<sup>[8,9]</sup>. For instance, activated microglia release of pro-inflammatory mediators including, cytokines [e.g., interleukin (IL)-1 $\beta$ , IL-6]<sup>[10,11]</sup>, chemokines (e.g., monocyte chemoattractant protein-1/CCL2, interferon- $\gamma$  inducible protein 10/CXCL10)<sup>[11-13]</sup> and reactive oxygen species<sup>[14,15]</sup>. Microglia also modulate the inflammatory response by releasing anti-inflammatory cytokines such as IL-10<sup>[16]</sup> and transforming growth factor (TGF)- $\beta$ <sup>[17]</sup>. Controlled neuroinflammation is neuroprotective<sup>[18]</sup>, however, excessive or chronic neuroinflammation is neurotoxic, it contributes to neurodegeneration, and disrupts neuronal function<sup>[1]</sup>. For instance, microglial activation and neuroinflammation are present in neurodegenerative diseases, CNS infection and trauma, as well as psychiatric disorders. Indeed, emerging evidence suggests pharmacological modulation of microglia may be beneficial in treating certain CNS disorders<sup>[19-22]</sup>.

Much has been discovered about microglia function, including inflammatory signaling, using *in vitro* approaches with primary cell cultures and transformed cell lines<sup>[23-25]</sup>. Significant insights have been obtained about inflammatory signaling using either primary rat or mouse microglia<sup>[26-29]</sup> or transformed cell lines such as BV-2 murine microglial cells<sup>[26,28-30]</sup>. While many of the findings have been observed in primary human microglia<sup>[14,31,32]</sup>, not surprisingly, there have been differences observed<sup>[24]</sup>. For example, bacterial lipopolysaccharide (LPS) and interferon- $\gamma$  (IFN $\gamma$ ) are potent inducers of inflammatory signaling (e.g., cytokine/chemokine expression) in both mouse and human microglia<sup>[29,32-34]</sup>, whereas, IL-1 $\beta$  activates human, but not mouse microglia<sup>[10,11,35,36]</sup>. The lack of responsiveness of mouse microglia to IL-1 $\beta$  stimulation is likely a consequence of very low IL-1 receptor (IL-1R) expression<sup>[36]</sup>.

Therefore, while there has been a wealth of knowledge obtained about rodent-derived microglia, it remains critical to better understand inflammatory signaling in human microglia. Primary human microglia (fetal and adult) remain a necessary tool, but they are relatively difficult and expensive to obtain and use, thus, only a limited number of research groups have access, funds, and expertise to extensively use this approach. Primary human microglia are commercially available (e.g., ScienCell Research Laboratories, cat. #HM-1900) however, they are often in limited supply or not in stock. Therefore, there is increasing interest in immortalized human microglial cell lines as sustainable tools for studying microglia function<sup>[37-39]</sup>. The importance of human microglial cell lines extends beyond basic understanding of microglia function but also these cell lines serve as a platform for investigating the effects of pharmacologic agents on microglia, with an eye toward drug development.

Recently, a novel, immortalized human microglial cell line (C20) was introduced to the field; the cells maintain microglial morphology, express multiple cell surface microglia markers, and express proinflammatory cytokines following stimulation with tumor necrosis factor- $\alpha$  (TNF $\alpha$ )<sup>[37]</sup>. We are particularly interested in IL-1 $\beta$ -induced inflammatory signaling in microglia due to the established role of IL-1 $\beta$  in neuroinflammation<sup>[40-43]</sup>. Interestingly, most studies utilize LPS and/or IFN $\gamma$  to stimulate microglia

*in vitro*; and to a large extent this is because most *in vitro* studies utilize rodent cells and these are the stimuli to which they respond. Therefore, the primary objective of our investigation was to advance our knowledge of IL-1 $\beta$ -induced inflammatory signaling in human microglia using C20 human microglial cells.

## METHODS

### Cells

C20 human microglial cells were obtained from David Alvarez-Carbonell, PhD (Case Western Reserve University) and details pertaining to the generation of this cell line were recently reported<sup>[37]</sup>. Briefly, these investigators obtained human microglia from ScienCell Research Laboratories, Carlsbad, CA (Cat# HM1900) and then immortalized the cells using simian virus 40 large T antigen and hTERT (to facilitate expression of human telomerase reverse transcriptase)<sup>[37]</sup>. The C20 cells that we obtained were confirmed to be of human origin by the Human Identity Testing Laboratory at Oklahoma State University Center for Health Sciences, which utilized the PowerPlex® 21 System (Promega, Madison, WI), a multiplex short tandem repeat system for human identification, as previously described<sup>[44]</sup>. For our experiments, cells were used at passages 5-10 and were either seeded in 24-well plates ( $1 \times 10^5$  cells/well) or in 100 mm dishes ( $3 \times 10^6$  cells) depending on the experiment and cultured in growth medium [Dulbecco's Modified Eagle Medium/Ham's F-12 50/50 mix supplemented with 2.5 mmol/L L-glutamine (Corning 10-090-CV), 10% fetal bovine serum (Atlanta Biologicals S11550), and 1% penicillin/streptomycin (Lonza 17603E)] until 90% confluent (4-5 days). Medium was replaced with serum-free medium (SFM) 24 h prior to stimulation. Normal human astrocytes (NHA, ScienCell, #HA1800) were maintained as previously described<sup>[45]</sup>.

### Stimulus

C20 were stimulated in SFM containing either LPS (*E. coli* K12, 1  $\mu$ g/mL; InvivoGen, San Diego, CA) or human recombinant IL-1 $\beta$  (20 ng/mL; Peprotech, Rocky Hill, NJ) for 10 min - 24 h depending on the specific experiment; whereas, NHA were stimulated with IL-1 $\beta$  (3 ng/mL) for 24 h in the single study in which they were used. Details regarding the number of independent experiments and replicate treatments within each experimental run are provided in the figure legends.

### Expression of microglial marker

While the precise function of transmembrane protein 119 (TMEM119) has yet to be determined, it is increasingly recognized as a reliable marker of human microglia that discriminates microglia in the brain from blood-derived macrophages<sup>[46-49]</sup>. We assessed TMEM119 expression by western blot analysis and fluorescent immunocytochemistry. For western blot analysis, whole cell lysates were collected from unstimulated and IL-1 $\beta$ -stimulated C20 cells (cultured in 100 mm dishes), using Triton X-100 lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100) containing MS-SAFE protease/phosphatase inhibitor (Sigma-Aldrich). Briefly, cells were rinsed with cold phosphate buffered saline (PBS), then lysed in 300  $\mu$ L of lysis buffer and collected into 1.5 mL tubes. The lysates were then incubated on ice for 45 min with intermittent mixing by inversion. Lysates were centrifuged for 10 min at  $20,800 \times g$  and 4 °C. The supernatant, containing whole cell protein was then collected and stored at -80 °C. Thirty micrograms of total protein were loaded on 7.5% polyacrylamide gels (BioRad TGX FastCast Acrylamide kit, #161-0171), electrophoresed and then transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were then incubated at 4 °C for 15-18 h with rocking. Membranes were blocked with 5% bovine serum albumin (BSA) for 2 h prior to incubation with antibodies. Primary antibodies included, anti-TMEM119 (1:100, Sigma #HPA051870), anti-gial fibrillary acidic protein (GFAP; 1:5000, Millipore #MAB360), and anti- $\beta$ -tubulin (1:1000, Cell Signaling #2146S). Anti-rabbit IgG, AP-linked (1:1000, Cell Signaling #7054S) was used as the secondary antibody. Whole cell lysates from NHA were used for comparison. Restore western blot stripping buffer (Thermo Scientific #21059) was used to remove antibodies and allow for re-labelling of membranes. The blots were scanned in a phosphorimager Typhoon 9410 (GE Healthcare, Uppsala, Sweden) using Amersham ECF Substrate #RPN-5785, and Image J (National Institutes of Health) was used for densitometric analysis.

TMEM119 expression was also assessed by fluorescent immunocytochemistry. Briefly, C20 cells were seeded on glass coverslips in 6-well dishes and cultured as described above until 70%-80% confluent. Following the treatment period (24 h), cells were washed three times with PBS then fixed in 4% paraformaldehyde for 15 min. Cells were washed with PBS and then incubated in blocking buffer (1% BSA in PBS) overnight at room temperature. Cells were then incubated in anti-TMEM119 (1:500 in PBS containing 0.5% BSA, Sigma #HPA051870) overnight at 4 °C with rocking. After washing with PBS, cells were incubated for 2 h in Alexa Fluor® 488 donkey anti-rabbit antibody (1:3000 in PBS containing 0.5% BSA; Life Technologies #A-21206). Cells were washed again with PBS, treated with 300 nmol/L 4',6-diamidino-2-phenylindole (DAPI; Sigma) without rocking for 15 min at room temperature, then washed in PBS prior to mounting in Prolong Gold anti-fade reagent (Invitrogen; Eugene, OR).

### Cytokine/chemokine expression

Levels of secreted CXCL10, CCL2, and IL-6 were measured in the culture medium using standard dual-antibody solid phase immunoassay (ELISA) kits purchased from Peprotech. Cytokine/chemokine concentrations were normalized to total cellular protein levels, which were determined using the bicinchoninic acid protein assay as previously described<sup>[50]</sup>.

### Expression of inflammatory signaling molecules

Induction of inflammatory signaling was determined by measuring phosphorylation of inhibitory kappa B alpha (I $\kappa$ B $\alpha$ ), and p38 mitogen-activated protein kinase (p38 MAPK) in cytoplasmic fractions; and phosphorylation of nuclear factor (NF)- $\kappa$ B p65 in nuclear fractions. More specifically, after experimental treatments, cells in 100 mm dishes were washed twice with PBS and nuclear and cytoplasmic protein extracts prepared as previously described<sup>[51]</sup>, with the exception of including 1 mmol/L sodium orthovanadate (Sigma, #450243) in the lysis buffers. Thirty micrograms of total protein were loaded on 7.5% polyacrylamide gels (BioRad TGX FastCast Acrylamide kit, #161-0171), electrophoresed and then transferred to PVDF membrane. Membranes were then incubated at 4 °C for 15-18 h with rocking. Primary antibodies included anti-phospho-I $\kappa$ B $\alpha$  (1:100, Cell Signaling #2859S), anti-I $\kappa$ B $\alpha$  (1:1000, Cell Signaling #4812S), anti-phospho-p38 (1:500, Cell Signaling #9215S), anti-p38 (1:1000, Cell Signaling #9212S), anti-phospho-NF- $\kappa$ B p65 (1:1000, Cell Signaling #3033S), anti-NF- $\kappa$ B p65 (1:1000, Cell Signaling #4764S), and anti- $\beta$ -tubulin (1:1000, Cell Signaling #2146S). Anti-rabbit IgG, AP-linked (1:1000, Cell Signaling #7054S) was used as the secondary antibody and restore western blot stripping buffer was used to remove antibodies and allow for re-labelling of membranes. The blots were scanned and analyzed as described above in the section on microglia marker expression.

### Statistical analyses

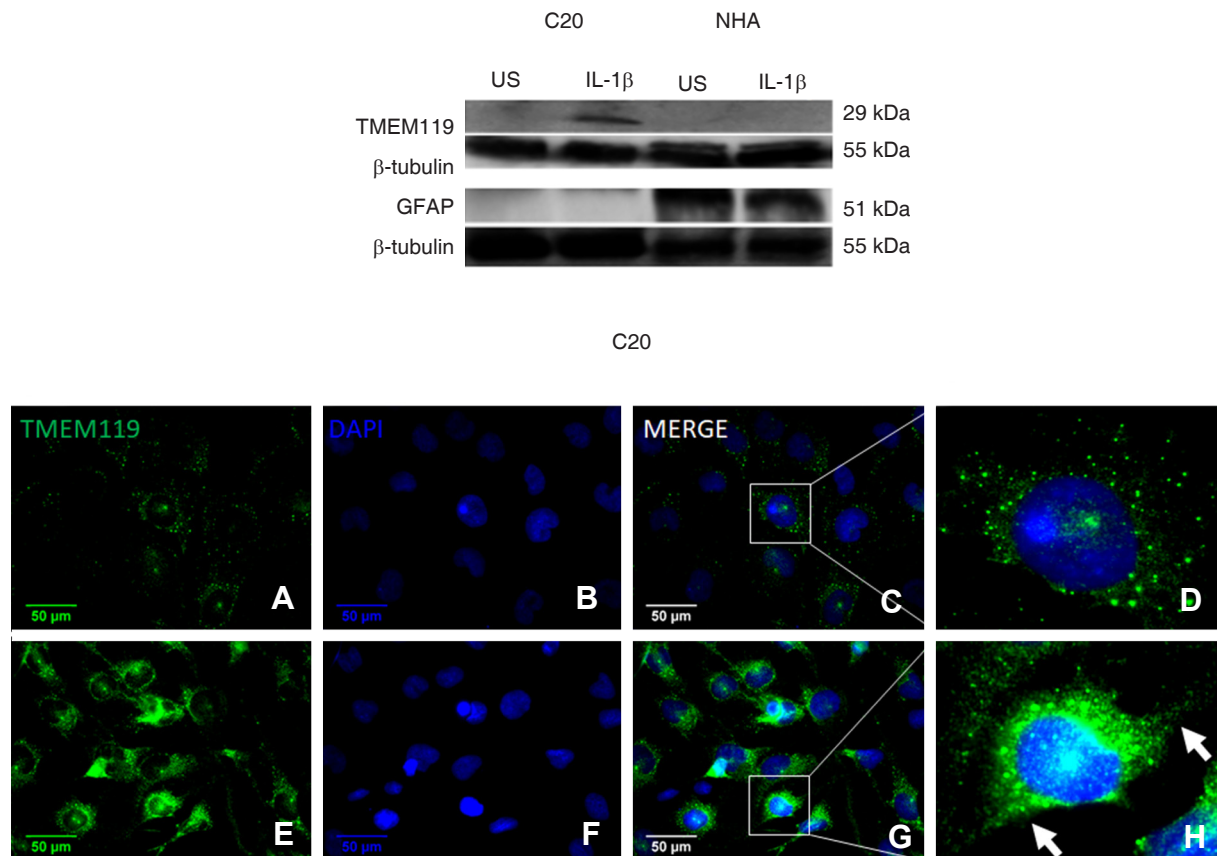
Prism version 7.0 software (GraphPad Inc., San Diego, CA) was used for figure presentation and statistical analysis. Analyses included one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. In those instances where data did not exhibit homogeneity of variance ( $P < 0.05$  for Bartlett's test), data were log-transformed prior to analysis. Data are presented as mean  $\pm$  SEM ( $n = 4-7$ ) and  $P < 0.05$  indicated statistically significant differences between groups.

## RESULTS

### Expression of microglial marker

TMEM119 was robustly expressed in IL-1 $\beta$ -treated C20 cells as indicated by a strong band at  $\approx$  29 kDa [Figure 1 Top]. Whereas, TMEM119 was not detected in either unstimulated C20 cells or in NHA by western blot analysis. As expected, the astrocyte marker, GFAP was expressed in NHA, but was not detected in C20 cells.

Using fluorescent immunocytochemistry we confirmed our western blot findings that IL-1 $\beta$ -treated C20 cells express TMEM119 [Figure 1 Bottom]. This immunocytochemistry approach revealed that TMEM119 is also expressed in unstimulated C20 cells. In both unstimulated and IL-1 $\beta$ -stimulated cells, TMEM119 was



**Figure 1.** C20 human microglial cells express the microglial marker transmembrane protein 119 (TMEM119). C20 cells were exposed to media alone (unstimulated; US) or media containing interleukin-1 $\beta$  (IL-1 $\beta$ ) (20 ng/mL) for 24 h. Top panel: western blot analysis was used to measure levels of TMEM119 and glial fibrillary acidic protein (GFAP) in whole cell lysates and  $\beta$ -tubulin was assessed as a loading control. Whole cell lysates from unstimulated and IL-1 $\beta$  (3 ng/mL)-stimulated normal human astrocytes (NHA) were used for comparison. The blots presented are representative of independent experiments ( $n = 3$  for C20; and  $n = 2$  for NHA). Bottom panel: fluorescent immunocytochemistry was used to further assess TMEM119 expression (green) in US (A-D) and IL-1 $\beta$ -stimulated (E-H) C20 cells; and nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI, blue). Images are shown at 400  $\times$  magnification. The arrows in box H highlight the cytoplasmic extensions

detected predominantly in the cytoplasmic and/or cell membrane regions [Figure 1 Bottom]. Consistent with the western blot findings, TMEM119 expression was more pronounced in the IL-1 $\beta$ -treated C20 cells compared to unstimulated cells.

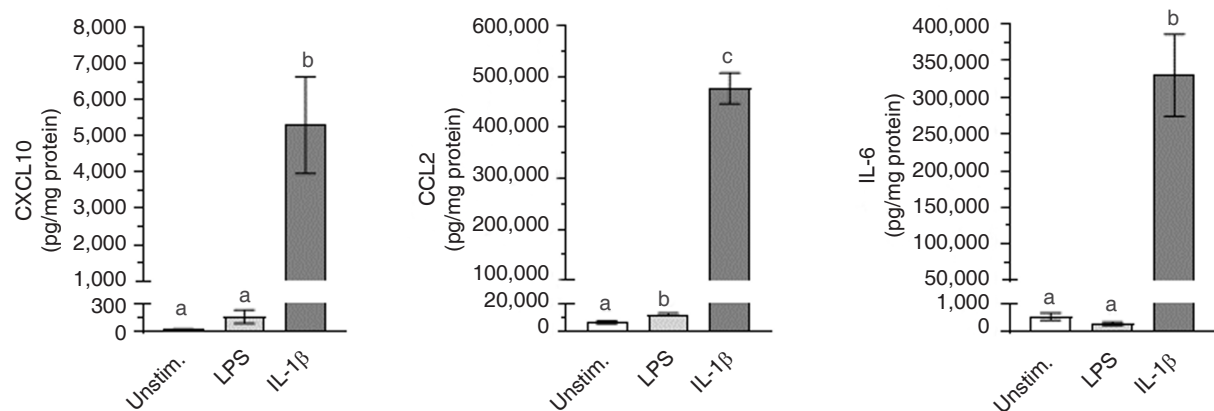
### Cytokine/chemokine expression

C20 cells constitutively expressed only minimal amounts of CXCL10, CCL2, and IL-6 [Figure 2]. However, stimulation with IL-1 $\beta$  significantly ( $P < 0.0001$ ) induced expression of CXCL10, CCL2, and IL-6. In contrast to IL-1 $\beta$ , LPS induced only a minimal, yet significant ( $P < 0.01$ ), increase in CCL2 expression, and did not significantly ( $P > 0.05$ ) affect CXCL10 or IL-6 expression.

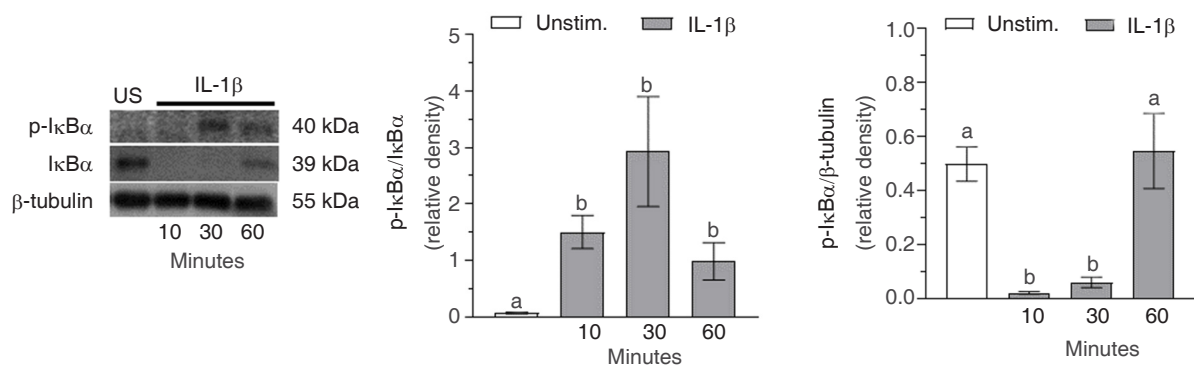
### Expression of inflammatory signaling molecules

Levels of phosphorylated I $\kappa$ B $\alpha$  in the cytoplasm were significantly ( $P < 0.0001$ ) increased after 10 min IL-1 $\beta$  exposure, remained elevated out to 60 min, but were beginning to drop toward baseline levels [Figure 3]. I $\kappa$ B $\alpha$  was constitutively expressed, with levels significantly ( $P < 0.0001$ ) reduced 10 min after IL-1 $\beta$  treatment. I $\kappa$ B $\alpha$  expression remained significantly ( $P < 0.001$ ) reduced 30 min after stimulation, but increased back to baseline levels by 60 min.





**Figure 2.** Cytokine/chemokine expression in C20 human microglial cells. C20 cells were exposed to media alone (unstimulated; Unstim.) or media containing either lipopolysaccharide (LPS) (1  $\mu$ g/mL) or interleukin-1 $\beta$  (IL-1 $\beta$ ) (20 ng/mL) for 24 h. CXCL10, CCL2, and IL-6 levels in the culture medium were determined by ELISA and normalized to total cellular protein (as determined by the bicinchoninic acid method). Data represent mean  $\pm$  SEM ( $n = 4-7$ ). Bars with different letters are significantly different ( $P < 0.01$ ) as determined by one-way ANOVA and Tukey's pairwise comparisons



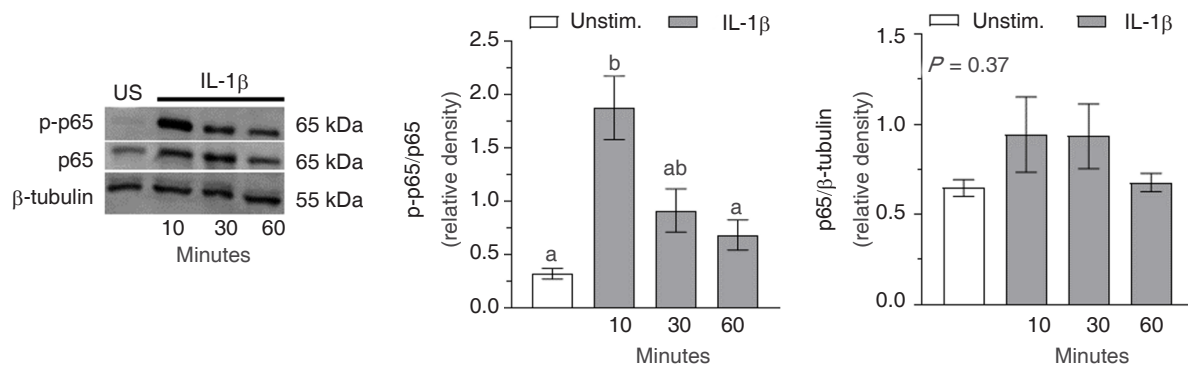
**Figure 3.** Interleukin-1 $\beta$  (IL-1 $\beta$ )-induced inhibitory kappa B alpha (IκBα) activation in C20 human microglial cells. C20 cells were exposed to media alone (unstimulated; US) or media containing IL-1 $\beta$  (20 ng/mL) for 10-60 min. Western blot analysis was used to measure levels of p-IκBα, IκBα, and β-tubulin in cytoplasmic protein extracts. The blots presented are representative of independent experiments ( $n = 4-5$ ) and the data represent mean  $\pm$  SEM. Bars with different letters are significantly different ( $P < 0.05$ ) as determined by one-way ANOVA and Tukey's pairwise comparisons

The expression of phospho-p65 in the nucleus rapidly increased within 10 min in response to IL-1 $\beta$  treatment ( $P < 0.0001$ ), before declining to baseline levels by 30 min [Figure 4]. Constitutive expression of p65 in the nucleus of C20 cells was evident and levels remained unchanged ( $P = 0.37$ ) throughout the 60 min exposure to IL-1 $\beta$ .

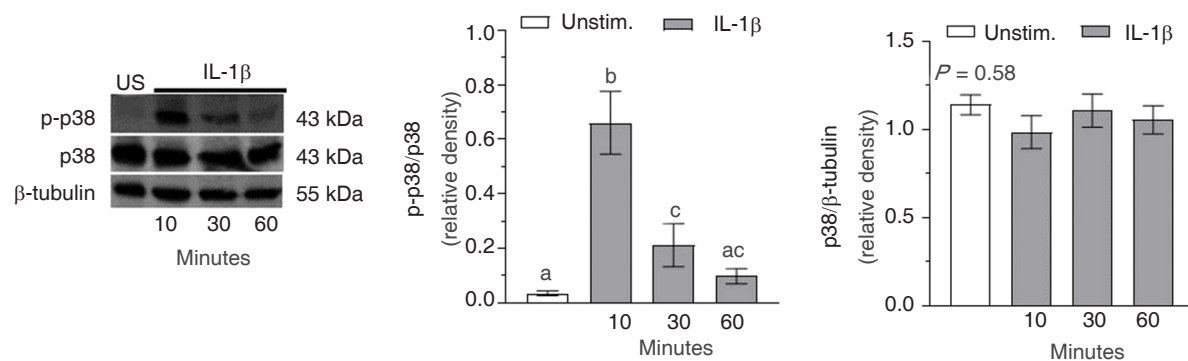
Phosphorylation of p38 in the cytoplasm occurred rapidly following treatment with IL-1 $\beta$  as indicated by a significant ( $P < 0.0001$ ) increase in phospho-p38/p38 after 10 min [Figure 5]. The levels of phospho-p38 then rapidly declined by 30 min ( $P < 0.05$ ), and returned to baseline by 60 min ( $P = 0.19$ ). Constitutive expression of p38 in the cytoplasm of C20 cells was robust and the expression levels remained constant ( $P = 0.58$ ) throughout the 60 min exposure to IL-1 $\beta$ .

## DISCUSSION

Microglia are a key component of the innate immune system with critical roles in response to injury and infection<sup>[1]</sup>. Furthermore, microglia are instrumental in neurodevelopment and physiological functions necessary for maintaining CNS homeostasis<sup>[52]</sup>. Microglia have been implicated in a multitude of CNS disorders, and modulation of microglia function has emerged as a potential therapeutic strategy<sup>[8]</sup>.



**Figure 4.** Interleukin-1 $\beta$  (IL-1 $\beta$ )-induced nuclear factor (NF)- $\kappa$ B p65 activation in C20 human microglial cells. C20 cells were exposed to media alone (unstimulated; US) or media containing IL-1 $\beta$  (20 ng/mL) for 10-60 min. Western blot analysis was used to measure levels of p-NF- $\kappa$ B p65, NF- $\kappa$ B p65, and  $\beta$ -tubulin in nuclear protein extracts. The blots presented are representative of independent experiments ( $n = 4-5$ ) and the data represent mean  $\pm$  SEM. Bars with different letters are significantly different ( $P < 0.05$ ) as determined by one-way ANOVA and Tukey's pairwise comparisons



**Figure 5.** Interleukin-1 $\beta$  (IL-1 $\beta$ )-induced p38 MAPK activation in C20 human microglial cells. C20 cells were exposed to media alone (unstimulated; US) or media containing IL-1 $\beta$  (20 ng/mL) for 10-60 min. Western blot analysis was used to measure levels of p-p38 mitogen-activated protein kinase (p38 MAPK), p38 MAPK, and  $\beta$ -tubulin in cytoplasmic protein extracts. The blots presented are representative of independent experiments ( $n = 5$ ) and the data represent mean  $\pm$  SEM. Bars with different letters are significantly different ( $P < 0.05$ ) as determined by one-way ANOVA and Tukey's pairwise comparisons

Altogether, there is substantial interest in further understanding microglia function. Much of what is currently known about microglia, stems from *in vitro* studies using rodent microglia, particularly immortalized cell lines. However, there is increasing interest in the use of immortalized human microglial cell lines to advance our understanding of human microglia function and discovery of pharmacological agents that modulate microglia<sup>[37,38,53,54]</sup>.

Among the very few immortalized human microglial cell lines available for use is the C20 human microglial cell line recently developed by Garcia-Mesa *et al.*<sup>[37]</sup>. These investigators utilized RNA sequencing to confirm the microglial phenotype of C20 cells and they demonstrated that these cells maintain migratory capacity and phagocytic activity characteristic of microglia<sup>[37]</sup>. Furthermore, C20 cells express numerous microglial surface markers, including cluster of differentiation (CD)11b, CD68, TGF $\beta$  receptor (TGF $\beta$ R), and the P2 purinergic receptor, P2RY12, as indicated by immunofluorescence and flow cytometry<sup>[37]</sup>. While CD11b<sup>+</sup> macrophages are also present in peripheral tissues<sup>[55]</sup>, TGF $\beta$ R and P2RY12 have been suggested to be microglial specific<sup>[56,57]</sup>. TMEM119 has also recently emerged as a microglia marker capable of discriminating between resident microglia and peripheral macrophages<sup>[46,47]</sup>, yet the functional importance of this protein remains to be elucidated. We have demonstrated for the first time that C20 cells express TMEM119. Interestingly, TMEM119 protein expression was greatest in C20 cells that were stimulated with IL-1 $\beta$ . While we did detect very small amounts of TMEM119 in unstimulated C20 cells during preliminary experiments, a substantial amount of total protein had to be loaded into the gels in order to achieve these

results. Whereas, when 30 µg of protein from unstimulated C20 cells were electrophoresed, we did not detect TMEM119. Further investigation is warranted to fully appreciate the expression profile of TMEM119 at the mRNA and protein levels; and the modulatory influence of proinflammatory mediators. Additional insights into the expression profile and functional role of TMEM119 in microglia are therefore needed and C20 cells may prove to be a useful tool in this line of investigation.

Previously, during the initial characterization of this cell line, C20 cells were found to secrete proinflammatory cytokines following stimulation with TNFα<sup>[37]</sup>. We have added to these findings and advanced our understanding of inflammatory signaling in C20 cells. More specifically, we provide the first evidence that IL-1β potently induces expression of CXCL10, CCL2, and IL-6 in C20 cells. We have also demonstrated that LPS differentially affects expression of these inflammatory mediators as evidenced by stimulation of CCL2 expression, but not CXCL10 or IL-6. Overall, it is clear that IL-1β is a much more effective inducer of cytokine/chemokine expression in C20 cells compared to LPS. In contrast, mouse microglia (including the BV-2 cells) are very responsive to LPS, but not to IL-1β due to the absence of IL-1R expression<sup>[36]</sup>.

IL-1β levels are elevated in a range of CNS disorders and this proinflammatory cytokine has been implicated as a key mediator of neuropathology<sup>[40]</sup>. Thus, it is fundamentally important to fully understand IL-1β-induced inflammatory signaling in human microglia. Furthermore, advancing our understanding of these signaling events in C20 human microglia is critical for developing this research tool. Secretion of CXCL10, CCL2 and IL-6 by C20 microglia in response to IL-1β exposure is a functionally relevant endpoint measure given both the important neurophysiological and neuropathological roles of these cytokines/chemokines. For instance, CXCL10 is initially neuroprotective in viral infections<sup>[58]</sup>, but can also contribute to neuropathology as evidenced in human immunodeficiency virus (HIV)-induced dementia<sup>[59,60]</sup>. CXCL10 also plays a role in neuropathology associated with traumatic brain injury (TBI)<sup>[61]</sup>, and emerging data suggest CXCL10 is involved in sickness behavior<sup>[62]</sup>. CCL2 functions as a chemotactic cytokine, activating and directing migration of numerous cell types<sup>[61]</sup>; and it is increasingly evident that increased levels of this chemokine in the brain contribute to neuropathology of HIV-dementia, AD, ischemia, epilepsy, and TBI<sup>[61]</sup>. The cytokine IL-6 acts in the hypothalamus as a regulator of metabolism<sup>[63]</sup> and has gained attention for its involvement in autism<sup>[64]</sup>, major depression<sup>[65,66]</sup>, and neurodegenerative diseases<sup>[67-69]</sup>. Therefore, C20 microglia are expected to be a useful tool in discovery of pharmacologic agents that may modulate microglial activation and subsequent release of proinflammatory factors.

We also provide the first evidence that IL-1β induces activation of key proinflammatory signaling molecules in C20 cells, including IκBα, NF-κB p65, and p38 MAPK. The importance of these signaling molecules in microglia activation is well established and these proteins are viable targets for pharmacologic modulation of inflammation<sup>[29,34,70-73]</sup>. Therefore, by demonstrating that these signaling pathways are functional in C20 cells, it is expected that these cells will be instrumental in the identification and characterization of novel pharmacologic agents intended to alter microglial function.

In conclusion, we have determined that IL-1β-activated C20 microglia express the microglia specific marker TMEM119. Additionally, we have provided the first evidence that IL-1β induces activation of IκBα, NF-κB p65, and p38 MAPK and subsequent secretion of CXCL10, CCL2 and IL-6 in C20 human microglia. These findings support the use of this human microglial cell line as a research tool to advance our understanding of microglia function and for the development of pharmacotherapies targeting a range of neuropathologies.

## DECLARATIONS

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### Authors' contributions

Concept, experimental design, literature review, statistical analysis, manuscript preparation: Davis RL

Performed experiments, data acquisition and analysis, and manuscript editing: Buck DJ

Performed experiments, experimental design, data acquisition and analysis, and manuscript editing: McCracken K

Performed experiments, data acquisition, and manuscript editing: Cox GW

Performed experiments, data acquisition and analysis, and manuscript editing: Das S

### Availability of data and materials

The raw data and materials are housed in the laboratory of Davis RL and available as appropriate.

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### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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# AUTHOR INSTRUCTIONS

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## 1. Submission Overview

Before you decide to publish with us, please read the following items carefully and make sure that you are well aware of Editorial Policies and the following requirements.

### 1.1 Topic Suitability

The topic of the manuscript must fit the scope of the journal. Please refer to Aims and Scope for more information.

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The journal adopts Gold Open Access publishing model since its establishment and has been distributing contents under Attribution 4.0 International License since October 2017, whereas Attribution-NonCommercial-ShareAlike 3.0 Unported had been adopted by then. Please make sure that you are well aware of these policies.

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All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smooth and efficient.

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## 2. Submission Preparation

### 2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, etc.

Here is a guideline of a cover letter for authors' consideration:

In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, etc.) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;

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Manuscript Type	Definition	Abstract	Keywords	Main Text Structure
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Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author includes an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	None required	None required	/
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Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	Unstructured abstract. No more than 150 words.	3-8 keywords	/

## 2.3 Manuscript Structure

### 2.3.1 Front Matter

#### 2.3.1.1 Title

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protein names are included, the abbreviated name rather than full name should be used.

### **2.3.1.2 Authors and Affiliations**

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

### **2.3.1.3 Abstract**

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### **2.3.1.4 Keywords**

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

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Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

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The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

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Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

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This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

### **2.3.2.5 Conclusion**

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

## **2.3.3 Back Matter**

### **2.3.3.1 Acknowledgments**

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Each author is expected to have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data, or the creation of new software used in the work, or have drafted the work or substantively revised it.

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Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. <i>Hypertension</i> 2002;40:679-86. [PMID: 12411462]



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Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. <i>J Appl Clin Pediatr</i> 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. <i>BJOG</i> 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: <a href="https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm">https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm</a> . [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. <i>Proc Natl Acad Sci U S A</i> . Forthcoming 2002.

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General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

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